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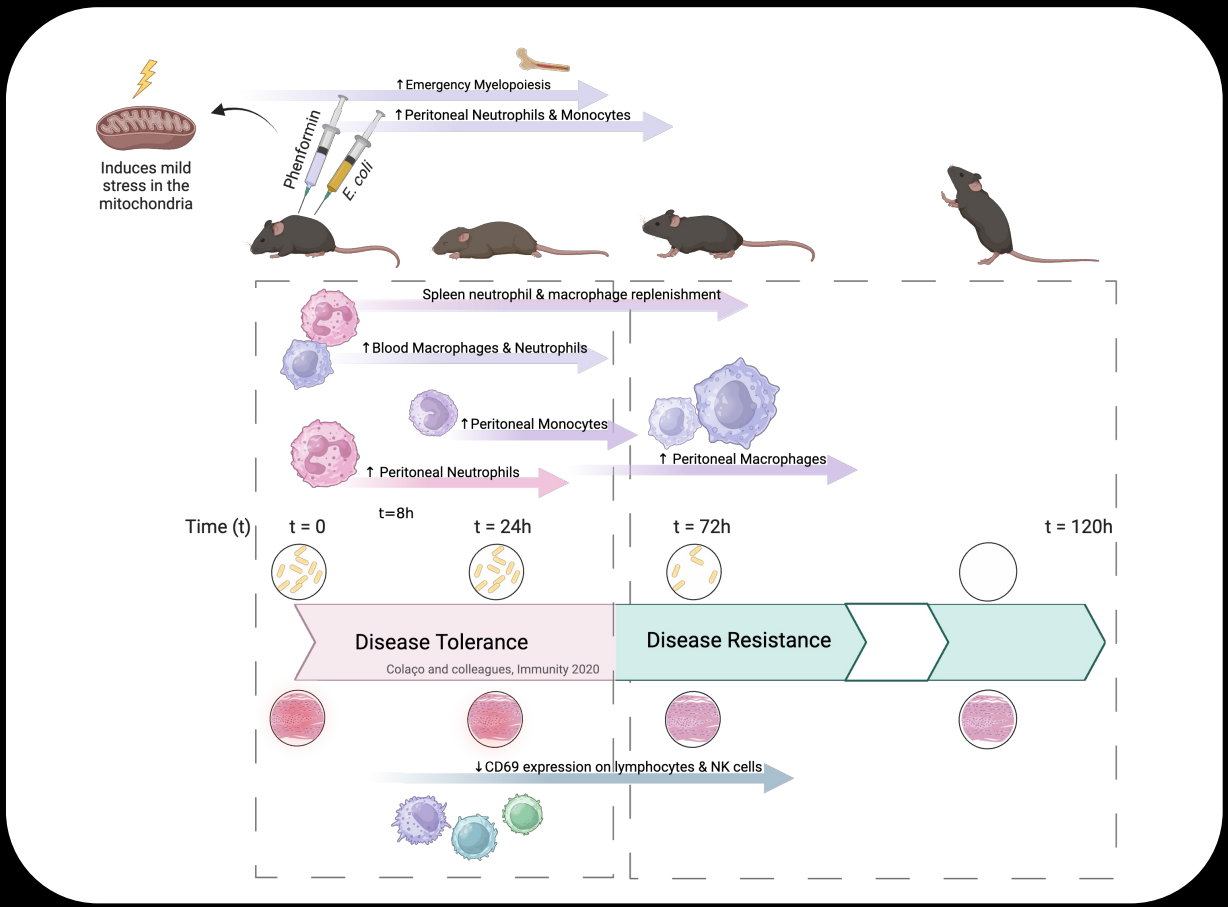
Contribution of resistance and disease tolerance mechanisms in the response against infection

Kátia Ribeiro de Jesus



Dissertation presented to obtain the **Ph.D degree in**

Integrative Biology and Biomedicine

Oeiras, August, 2024

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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Cover: Illustration of the working model hypothesized based on the findings of this PhD dissertation. Mechanisms of disease tolerance and resistance conferring protection by mild mitochondrial stress during infection. Original illustration created in Biorender. Illustration credits: Kátia Jesus.

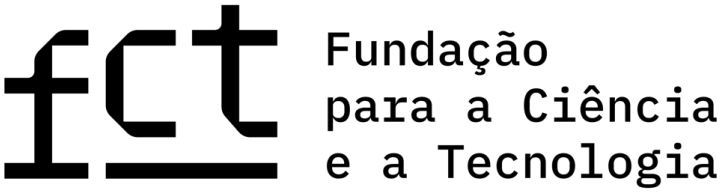

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Table of contents

Acknowledgments ..... **V**
Summary ..... **IX**
Sumário ..... **XI**
List of abbreviations ..... **XIII**
List of figures ..... **XVI**
Chapter 1
Introduction
1.1. Immunity and infection **1**
1.1.1. The immune system 1
1.1.1.1. Innate and adaptive immunity 1
1.1.1.2. Communication mechanisms in the immune system 3
1.1.2. Host-pathogen interactions: sensing mechanisms 4
1.1.2.1. Microbe-associated molecular patterns (MAMPs) sensing via Pattern recognition receptor (PRRs) 4
1.1.2.2. The Danger model: sensing of damage-associated molecular patterns (DAMPs)…… 5
1.1.2.3. Effector-triggered immunity (ETI) 5
1.2. Hematopoiesis in homeostasis and during infection **7**
1.2.1. Emergency myelopoiesis and trained immunity 9
1.3. The role of inflammation in infection **10**
1.4. Sepsis, a maladaptive host response to an infection **11**
1.4.1. The pathophysiology of sepsis 11
1.4.2. Current diagnosis, therapeutic strategies, and caveats 13
1.5. Finding the balance: disease tolerance and resistance mechanisms **14**
1.6. Stress responses in the maintenance of organismal homeostasis **16**
1.6.1. The role of hormesis in infection 18
1.7. The organismal roles of the mitochondria **20**
1.7.1. Bioenergetic processes 21
1.7.2. Signaling and cell death 22
1.7.3. Targeting the mitochondrial ETC 24
1.7.4. The impact of mitochondrial fitness on immune cells 24
1.8. Thesis aims and outline **28**
1.9. References **30**
Chapter 2

Phenformin protects against bacterial sepsis by activating host-dependent disease tolerance and resistance mechanisms

2.1. Abstract 40

2.2. Introduction 40

2.3. Methods 42

2.3.1. Experimental Models 42

2.3.1.1. Mice 42

2.3.1.2. Bacterial cultures 42

2.3.2. Methods details 43

2.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments….. 43

2.3.2.2. Colony forming units (CFUs) assay 44

2.3.2.3. Histopathology 44

2.3.2.4. Biochemical assays of mouse serum 45

2.3.2.5. E. coli growth curves *in vitro* 45

2.3.2.6. Data modeling and statistical analysis 45

2.4. Results 46

2.4.1. Bacterial peritonitis model optimization for mild sepsis using E. *coli* 46

2.4.2. Phenformin-induced mild mitochondrial stress confers protection against bacterial sepsis….. 48

2.4.3. Disease resistance and tolerance mechanisms confer protection to phenformin-treated septic mice 49

2.4.4. Phenformin does not impact *E. coli* infection capacity 52

2.4.5. Phenformin gluconeogenesis suppressive capacity is normalized within 8 h of treatment… 54

2.5. Discussion 56

2.6. References 59

2.8. Supplementary data 61

Chapter 3

Phenformin-induced modulation of the immune response is organ- and cell-type specific

3.1. Abstract 63

3.2. Introduction 63

3.3. Methods 66

3.3.1. Experimental Models 66

3.3.1.1. Mice 66

3.3.1.2. Bacterial cultures 66
3.3.2. Methods details 66
3.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments….. 66
3.3.2.2. Flow cytometry sample preparation 66
3.3.2.3. Flow cytometry staining 68
3.3.2.4. Data modelling and statistical analysis 70
3.4. Results 70
3.4.1. Mild mitochondrial stress modulates cellular dynamics during infection 70
3.4.2. Organ-specific innate immune cell recruitment is promoted by phenformin treatment of septic mice 73
3.4.3. Phenformin-modulated lymphoid cell migration 82
3.4.4. Adaptive immune cell activation is controlled by phenformin treatment of septic mice……. 83
3.5. Discussion 85
3.6. References 90
3.7. Supplementary data 94

Chapter 4

Contribution of phenformin-induced immune cell population changes to tolerance and resistance mechanisms

4.2. Introduction 104
4.3. Methods 106
4.3.1.2. Primary cell culture of bone marrow-derived macrophages (BMDMs) 106
4.3.1.3. Bacterial cultures 107
4.3.2. Methods details 107
4.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments….. 107
4.3.2.2. Colony forming units (CFUs) assay 107
4.3.2.3. Biochemical assays of mouse serum 107
4.3.2.4. Adoptive cell transfer experiments 108
4.3.2.5. CD8 T cell negative magnetic isolation 108
4.3.2.6. Flow cytometry sample preparation 109
4.3.2.7. Flow cytometry staining 109
4.3.2.8. BMDM infection clearance assay 111
4.3.2.9. Data modeling and statistical analysis 112
4.4. Results 113

4.4.1. Phenformin does not appear to skew T helper cell differentiation fate during infection… 113
4.4.2. Damage marker profiles cannot be replicated by adoptive cell transfers from infected donor mice to naïve recipient mice 114
4.4.3. Depletion of T lymphocyte population does not decrease sepsis-associated tissue damage or phenformin protection 117
4.4.4. Phenformin-induced myelopoiesis as a booster for the immune system against infection… 119
4.4.5. BMDM functional capacity is not greatly increased by phenformin 124
4.5. Discussion 125
4.6. References 129
4.7. Supplementary data 131
Chapter 5
General discussion and future perspectives 134
5.1. References 144
Appendix 146

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Summary

Organismal homeostasis is maintained through highly complex and tightly regulated mechanisms that ensure equilibrium, independently of external challenges. Extreme challenges can disrupt this equilibrium, leading to the development of deleterious conditions, such as autoimmunity, cancer or chronic inflammation. A prime example of a condition imposing extreme organismal challenges is sepsis. Defined as an acute inflammatory condition arising from an infection by any type of pathogen, sepsis is hallmarked by a dysregulated host response that can fail to resolve the infection while, in parallel, inducing extensive tissue damage to the host. This is because the condition of sepsis results from extreme deviations of the host immune response from the homeostatic balance, wherein resistance and tolerance responses become erroneously orchestrated.

The concept of hormesis demonstrates that small stresses can be beneficial, as they induce cellular and organismal adaptation, which in turn will provide protection against more severe insults. In fact, a growing body of evidence indicates that harnessing stress responses can promote protection by inducing disease tolerance mechanisms and reducing infection-driven damage, even with demonstrated important roles in lifespan extension. Our lab has previously demonstrated that mild mitochondrial stress can induce strong protection against infection in a severe mouse model of sepsis. This protection was reported to be conferred by disease tolerance mechanisms through the reduction of tissue and organ damage imposed by the infection. The work developed under the scope of this thesis set out to understand the contribution of tolerance and resistance mechanisms during infection, hereby investigating the impact of mild mitochondrial stress conferred by phenformin administration in both defense mechanisms. Ultimately, we aim to achieve a better understanding of the mechanisms that underlie the protective programs that could confer hormesis by mild mitochondrial stress.

We began by questioning whether mild mitochondrial stress only induced tolerance mechanisms or whether it could be a platform to reestablish the appropriate balance between tolerance and resistance responses to infection. Our results demonstrate that mild mitochondrial stress protection in sepsis is at first conferred by the prioritization of disease tolerance mechanism, followed by a switch and a promotion of resistance responses. By characterizing the immune population dynamic changes imposed by phenformin treatment we demonstrated downregulation of CD69 in lymphocytes and NK cells during early time points of infection. On the other hand, we have observed rapid recruitment of neutrophils and monocytes to the peritoneal cavity and the faster recovery of tissue-resident macrophage populations associated with the treatment of infected mice.

We hypothesize that the reduction of CD69 in lymphoid populations could indicate a modulation of these cells' proliferative, effector, and metabolic function by phenformin. This could prevent their over-activation. Since lymphoid cells constitute key players in promoting tissue damage and organ dysfunction, this could represent a cellular basis for the early-on prioritization of disease tolerance mechanisms. The augmented recruitment of innate immune cells to the site of infection initiation strongly suggests a modulation of innate immune cells for the promotion of resistance responses. Indeed, phenformins' ability to modulate hematopoiesis and promote emergency myelopoiesis, which is reflected in neutrophils and monocyte differentiation and recruitment, even in the absence of the infection insult, suggests that treatment might confer protection by inducing a pre-emptive boost of myelopoiesis, which upon the bigger challenge, the infection, will have a protective role. This should be subject to further investigation as a possible trained immunity mechanism.

Taken together, our results support the crucial role mitochondria play in orchestrating host-pathogen interaction. The defense mechanisms elicited by mild mitochondrial stress demonstrated in this study are of great relevance and should be subject to more detailed investigation to clarify the mechanistic basis of the protective phenotypes harnessed by mitohormesis.

Sumário

A integridade da homeostase de um organismo é mantida através de mecanismos extremamente complexos e altamente controlados que garantem o equilíbrio do mesmo, independentemente de estímulos externos. Os estímulos de maior severidade podem perturbar este equilíbrio, levando ao desenvolvimento de condições deletérias, como autoimunidade, cancro ou inflamação crónica. Um exemplo clássico de uma condição que impõe estímulos de maior severidade ao organismo é a sépsis. Esta é definida como uma condição de inflamação aguda derivada de uma infeção por qualquer tipo de agente patogénico. A condição de sépsis é marcada por uma resposta desregulada do hospedeiro que pode resultar na falha da resolução da infeção e, em paralelo, na indução de danos extensos aos tecidos e órgãos do hospedeiro. Isto ocorre porque a condição de sépsis resulta de alterações extremas do equilíbrio homeostático da resposta imune do organismo, durante os quais as respostas de resistência e tolerância são orquestradas de maneira errónea.

O conceito de hormese procura demonstrar que pequenas perturbações celulares podem ser benéficas, uma vez que levam à adaptação celular e do organismo, o que, por sua vez, providenciam proteção a uma agressão mais severa. Na realidade, um número crescente de estudos indica que a indução de respostas a perturbações celulares pode gerar proteção através da indução de mecanismos de tolerância, reduzindo danos derivados das infeções e até mesmo promovendo mecanismos que influenciam a extensão do tempo de vida. O nosso laboratório demonstrou anteriormente que uma perturbação mitocondrial ligeira pode induzir uma forte proteção contra infeção num modelo de murganho de sépsis severa. Foi possível confirmar que esta proteção é conferida por mecanismos de tolerância através da redução de danos causados pela infeção nos tecidos e órgãos. O trabalho desenvolvido no âmbito desta tese, procurou compreender em maior detalhe a contribuição dos mecanismos de tolerância e resistência durante a infeção, investigando o impacto de uma perturbação mitocondrial ligeira, através da administração de fenformina, em ambos os mecanismos de defesa. Procurando, em última análise, uma melhor compreensão dos mecanismos subjacentes aos programas de proteção que podem corroborar a promoção de hormese através de perturbações mitocondriais ligeiras.

Começámos por questionar se a perturbação mitocondrial ligeira estava apenas a originar uma indução de mecanismos de tolerância ou se poderia ser uma plataforma para restabelecer o equilíbrio apropriado entre as respostas de tolerância e resistência à infeção. Os nossos resultados demonstram que a proteção conferida pela perturbação mitocondrial ligeira à sépsis é iniciada através da priorização de mecanismos de tolerância, seguida por uma promoção de respostas de resistência. Através da caracterização das alterações na

dinâmica das populações de células imunes impostas pelo tratamento com fenformina, demonstrámos uma redução na expressão de CD69 em linfócitos e células NK durante a fase inicial da infeção. Por outro lado, observámos o rápido recrutamento de neutrófilos e monócitos para a cavidade peritoneal e a recuperação mais rápida das populações de macrófagos residentes associadas ao tratamento de murganhos infetados.

Tendo estes resultados em consideração, é possível especular que a redução do CD69 em populações linfóides poderá indicar uma modulação da função proliferativa, efetora e metabólica dessas células pela fenformina. Isso poderá levar a uma redução da ativação destas células imunes, que constituem os principais agentes de indução de danos aos tecidos e órgãos, representando uma possível justificação a nível celular da priorização dos mecanismos de tolerância. Em paralelo, o maior recrutamento de células imunes inatas para o local de início da infeção sugere uma modulação de células imunes inatas para priorização de respostas de resistência. De facto, a capacidade da fenformina de modular a hematopoiese e promover a mielopoiese de emergência que se reflete no aumento da diferenciação e recrutamento de neutrófilos e monócitos, mesmo na ausência do estímulo da infeção, sugere que o tratamento pode conferir proteção ao induzir preventivamente mielopoiese. Numa situação de uma agressão maior, como a infeção, isto reflete-se num mecanismo protetor, o qual necessita de investigação mais detalhada de modo a esclarecer se este se trata de um mecanismo de imunidade treinada.

Em resumo, os nossos resultados apoiam o papel crucial que as mitocôndrias têm na mediação da interação hospedeiro-patógeno. Os mecanismos de defesa eliciados pela perturbação mitocondrial ligeira demonstrados neste estudo são de grande relevância e devem ser sujeitos a uma investigação mais detalhada para esclarecer a base mecanística dos fenótipos protetores derivados da mitohormese.

List of abbreviations

ADP	Adenosine diphosphate
AIM2	Absent in melanoma-2
ALRs	AIM2-like receptors
ALT	Alanine transaminase
AMPK	5' AMP-activated protein kinase
AP-1	Activator protein 1
APCs	Antigen-presenting cells
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BMDMs	Bone marrow derived macrophage
C. elegans	*Caenorhabditis elegans*
Ca2+	Calcium ion
CFU	Colony-forming unit
CFP	Cyan fluorescent protein
CLRs	C-type lectin like receptors
cMoPs	Common monocyte progenitors
CMPs	Common myeloid progenitors
CO2	Carbon dioxide
CSF	Colony-stimulating factor
CT	Computerized tomography
D. melanogaster	*Drosophila melanogaster*
DAMPs	Danger-associate molecular patterns
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Emergency myelopoiesis
E. coli	*Escherichia coli*
ERG-1	Erythroblast transformation-specific related gene-1
ETC	Electron transport chain

ETI	Effector-triggered immunity
FACS	Fluorescence-activated cell scanning
FADH2	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FBS	Fetal bovine serum
FGF21	Fibroblast growth factor 21
GDF15	Growth differentiation factor 15
GLUT1	Glucose transporter 1
GM-CSF	Granulocyte-macrophages-CSF
GMPs	Granulocyte-monocyte progenitors
GP	Granulocyte progenitors
HSCs	Hematopoietic stem cells
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPMs	Large peritoneal macrophages
LPS	Lipopolysaccharide
LT-HSC	Long-term HSC
MAMPs	Microbe-associated molecular patterns
MDHR	Macrophage disturbance of homeostasis reaction
MDP	Monocyte-dendritic cell progenitors
MDR	Macrophage disappearance reaction
MHC	Major histocompatibility complex
MODS	Multi-organ dysfunction syndrome
MPP	Multipotent progenitor
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)
NETs	Neutrophil extracellular traps

NF-κb	Nuclear factor κb
NK	Natural killer
NLRs	NOD-like receptors
NOD	Nucleotide oligomerization domain
OD	Optical density
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffer saline
PRRs	Pattern recognition receptors
PTSD	post-traumatic stress disorder
RBC	Red blood cell
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure assessment
SPF	Specific pathogen-free
SPMs	Small peritoneal macrophages
StrepR	Streptomycin resistant
ST-HSC	Short-term HSC
T-ALL	T-cell acute lymphoblastic leukemia
TCA	Tricarboxylic acid
Th	T helper
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	T regulatory
UPR	Unfolded protein response

List of figures

Figure 1. 1 - Models of surveillance mechanisms for the initiation of immune response to an insult. 6
Figure 1. 2 - Hematopoiesis during homeostasis. 8
Figure 1. 3 - Dysregulated organismal, tissue and cellular pathways of resistance and tolerance during sepsis. 12
Figure 1. 4 – Disease resistance and tolerance strategies during infection and inflammation. 15
Figure 1. 5 - The concept of hormesis: dose-dependent cellular responses to stress. 18
Figure 1. 6 - Mitochondrial behaviors, structure and metabolism involved in the maintenance of cellular homeostasis by contributing to cellular dynamics, bioenergetics and signaling processes. 23
Figure 2. 1- Bacterial peritonitis model optimization. 47
Figure 2. 2 - Mild mitochondrial stress protects mice from mild sepsis. 49
Figure 2. 3 - Phenformin-induced resistance promotes pathogen clearance mechanisms. ..50
Figure 2. 4 - Phenformin-induced tissue protection results in lower tissue damage scores. .51
Figure 2. 5- Cytokine profiles in circulating blood during infection and treatment. 52
Figure 2. 6 - The infection capacity of *E. coli* is not impacted by phenformin. 54
Figure 2. 7 - Gluconeogenesis suppressive capacity of phenformin. 55
Supplementary Figure 2. 1 - Growth curves of the Streptomycin resistant *E. coli* strain in the presence or absence of 100, 200, 300, 400, 500 µg/mL phenformin. 61
Figure 3. 1 - Phenformin impacts viability and total cellularity across tissues. 72
Figure 3. 2 - Phenformin promotes neutrophil systemic recruitment to the peritoneum. 75
Figure 3. 3 - Macrophage and blood monocyte population homeostasis during infection is enhanced by phenformin. 77
Figure 3. 4 - Mitochondrial stress triggers macrophage disturbance of homeostasis reaction and promotes pre-emptive monocyte recruitment to the peritoneum. 79
Figure 3. 5 - Phenformin modulates immune cell recruitment to the lung during infection. ...81
Figure 3. 6 - Phenformin reduces lymphocyte and NK cell infection-driven activation. 84
Supplementary Figure 3. 1 - Phenformin modulates lymphocyte and NK cell migration during infection. 95
Supplementary Figure 3. 2 - Phenformin reduces lymphocyte and NK cell activation during infection across tissues. 97

Supplementary Figure 3. 3 - Gating strategy of activated CD4 T, regulatory T cells, CD8 T and B cells. Plots for spleen samples. Gating strategy in blood, lung and peritoneal lavage fluid follows the same strategy.....98
Supplementary Figure 3. 4 - Gating strategy of spleen activated NKT and NK cells. Plots for spleen samples. Gating strategy in blood, lung and peritoneal lavage fluid follows the same strategy. 99
Supplementary Figure 3. 5 - Gating strategy of blood neutrophils, CD11c+ dendritic cells, eosinophils, Ly6C monocytes and macrophages. 100
Supplementary Figure 3. 6 - Gating strategy of lung alveolar macrophages, neutrophils, immature macrophage-like cells and interstitial macrophages. 101
Supplementary Figure 3. 7 - Gating strategy of spleen neutrophils, CD11c+ dendritic cells, red pulp macrophages (RPM) and macrophages. 102
Supplementary Figure 3. 8 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, transitioning large peritoneal macrophages and large peritoneal macrophages. 102
Supplementary Figure 3. 9 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, transitioning large peritoneal macrophages and large peritoneal macrophages. 103
Figure 4. 1 - Phenformin does not significantly impact FoxP3 regulatory cells in the spleen. 113
Figure 4. 2 - Adoptive cell transfers of whole spleen cells do not replicate damage marker kinetics in naïve mice.....115
Figure 4. 3 - Adoptive cell transfer of CD8 T lymphocytes does not replicate circulating damage marker kinetics in naïve mice. 116
Figure 4. 4 - Phenformin conferred protection in mild sepsis is conserved in B6.Rag KO mice. 117
Figure 4. 5 – B6.Rag2 KO mice show similar levels of damage to wild-type animals. 118
Figure 4. 6 – Bacterial clearing in the absence of lymphocytes follows similar kinetics 119
Figure 4. 7 - Phenformin induces emergency myelopoiesis. 122
Figure 4. 8 - Neutrophil and monocyte phenformin-induced recruitment to the peritoneum. 123
Figure 4. 9- Phenformin does not increase bone-marrow derived macrophage clearing capacity. 124
Supplementary Figure 4. 1 - Vital parameter from adoptive cell transfer experiments of whole spleen. 131
Supplementary Figure 4. 2 - Vital parameter from adoptive cell transfer experiments of CD8 T lymphocytes. 131

Supplementary Figure 4. 3 - Gating strategy of bone marrow myeloid and lymphoid progenitors and fully committed cells.....132
Supplementary Figure 4. 4 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, large peritoneal macrophages and monocytes.....133
Figure 5. 1 – Mechanisms of protection conferred by mild mitochondrial stress during infection. 139

Chapter 1

Introduction

1.1. Immunity and infection

1.1.1. The immune system

Homeostasis consists of the ability of an organism to maintain internal processes and mechanisms in a dynamic equilibrium independently of external and internal conditions, thus ensuring survival and proper functioning. Besides physiological processes, such as thermoregulation, the immune system plays a central role in maintaining organismal homeostasis[1].

The immune system comprises several complex, tightly regulated, and coordinated groups of cells, tissues, and organs. These were, at first, only described to have important roles in responding to insults and in building up appropriate strategies to clear out threats and promote repair of damage driven by the insult, allowing for restoration of homeostasis [2,3]. However, a growing body of evidence shows that immune cells are also essential for sustaining life, as demonstrated by the role of the immune system in fetal development, microbiota equilibrium, metabolism, central nervous system and neuronal processes, and even the discovery of the importance of macrophages in electrical conduction for maintenance of the heartbeat [4–7]. Not surprisingly, immune system dysfunction, associated with extremely disruptive conditions or aging, is at the basis of many diseases or complex conditions that still lack appropriate treatment strategies, such as autoimmunity, cancer, neurodegenerative, and chronic inflammatory conditions[8–12]. Thus, it is important to understand the immune system, the different immune cell populations, their respective functioning and signaling mechanisms, how these populations are maintained, and the changes they undergo during an insult.

1.1.1.1. Innate and adaptive immunity

The most simplistic approach to understanding the immune system is to divide it into innate and adaptive immunity.

Herein, innate immunity is the most evolutionarily conserved arm of the immune system. It includes the physical and chemical barriers that provide protection from an insult. Upon breach of the integrity of these defenses, innate immune cells, complement, and acute phase proteins build up the defense mechanisms characterized by the generation of rapid, non-specific responses towards the insults[13]. Cells from the innate immune arm provide an acute and fast response to insults that is non-specific and relies on detection via innate immune cell pattern recognition receptors (PRRs) of signaling molecules released directly by the invading pathogens or of molecules resulting from the invasion of the pathogen in host cells. The

effector function of innate immune cells relies to a larger extent on endocytic and phagocytic processes for pathogen clearance, wherein key immune cell players are granulocytes, neutrophils, monocytes, macrophages, and dendritic cells[13].

On the other hand, adaptive immune responses are characterized by their specificity and hallmarked by a memory feature. Upon first encounter with an insult, the adaptive immune response has a lag time due to the requirement of mounting antigen-specific responses through affinity recombination in the germinal centers. However, upon re-exposure, this provides a faster and more targeted response. Adaptive immune cells include T and B lymphocytes, which can be subcategorized according to their function (T cytotoxic, T helper, or T regulatory, B memory or plasma cells for example)[13]. Another important feature of adaptive immune cells is the capacity to recognize self-antigens, which provides a mechanism to detect intracellular pathogens within host cells[14].

Activation of adaptive immune responses occurs through the actions of antigen-presenting cells (APCs, such as dendritic cells, macrophages, and even B cells), which phagocyte invading pathogens and present the foreign antigen to T cells to promote activation and proliferation. Here, CD8 cytotoxic T cells go through clonal expansion, leading to the production of effector cells whose mode of action is based on the release of substances that induce apoptosis of target cells; on the other hand, CD4 helper T cells mediate germinal center affinity maturation of antibody-producing B cells. Within T helper cells, Th1, Th2, and Th17 promote humoral responses to distinct pathogens and stimuli. Th1 cells are primarily involved in intracellular pathogen clearance, such as viruses and intracellular bacteria, Th2 respond to extracellular pathogens, such as helminths and parasites. In contrast, Th17 are involved in bacterial and fungal extracellular infection clearance mechanisms[15]. On the other hand, T regulatory cells are fundamental for controlling immune responses upon pathogen elimination, and they also have fundamental roles in suppressing aberrant responses to self-antigens, which can ultimately lead to autoimmune or degenerative conditions[13,14,16].

Natural killer (NK) cells were, for the longest time, characterized as lymphoid-origin innate immune cells due to their rapid and seemingly unspecific response to insults despite their common morphology and shared expression of many surface markers with lymphocytes. This view was challenged by the discovery that NK cells can distinguish self from non-self-antigen and that the environment can also influence their responses. Studies have shown that NK cells can mount antigen-specific responses and have an immunological memory[17–20]. As a result, NK cells can interact and function as both innate and adaptive immune cells, being involved in the orchestration of immune responses from both arms of the immune system.

Thus, NK cell classification and function remain controversial and in need for more detailed studies.

1.1.1.2. Communication mechanisms in the immune system

Appropriate immune responses required for maintaining homeostasis and during responses to insults result from proper structure and functioning of both arms of the immune system, but also, efficient communication between different cells. Here, cell-to-cell communication, mediated by direct contact or signaling molecules, plays a major role in the coordination of the immune system. In fact, aberrant communication can result in the development of pathological conditions, such as autoimmunity, chronic inflammation, or even cancer[21–23].

Upon infection, appropriate cell-to-cell communication orchestrates the response to neutralize and clear out the pathogen. Here, phagocytic cells, such as granulocytes, neutrophils, and macrophages, will contribute to a primary response to infection while, in parallel, promoting the recruitment of more immune cells to the site of infection. Major signaling molecules for cell recruitment during bacterial infections are tumor necrosis factor (TNF), interleukin 1 and 6 (IL-1, IL-6). During these early stages, APCs, such as long-lived macrophages and dendritic cells, initiate adaptive immune responses by presenting pathogenic antigens to T helper cells and promoting germinal center responses. These pro-inflammatory signals also modulate intracellular pathways, including kinases, caspases, transcription factors, and even metabolic profiles, ultimately reprogramming cells to deal with the thread[24–26].

In line with this, the control of immune responses during infection and restoration of homeostatic profiles also heavily depends on appropriate signaling between cells and organs, that modulate the rewiring from the pro-inflammatory to anti-inflammatory profile. Here, the production and secretion of anti-inflammatory cytokines such as interleukin 10 (IL-10) and tumor growth factor β (TGF-β) and the regulatory activity of Treg cells to inhibit inflammatory responses are essential for the induction of exhaustion markers to limit the activity of effector cells. For an efficient transition to resolution of inflammation and restoration of homeostasis after an insult, these changes need to occur in a systemic fashion[27,28].

The orchestration of an appropriate immune response to an insult strongly relies on detecting and identifying the invading agent. Thus, the immune system's sensing mechanisms have also been the subject of extensive study, and over the years, a list of surveillance recognition models has been described.

1.1.2. Host-pathogen interactions: sensing mechanisms

The most known and studied function of the immune system is to detect, identify, and neutralize threads to the organism. This detection can be based on direct cell-cell interaction or through signaling molecules that are released due to the infection and detected by patrolling immune cells, which, upon recognition, trigger appropriate immune responses. Despite being an extensively studied subject, a comprehensive model for immunological surveillance is yet to be defined. Currently, the following three main models of immune sensing are broadly accepted in literature:

1.1.2.1. Microbe-associated molecular patterns (MAMPs) sensing via Pattern recognition receptor (PRRs)

Following Metchinkoff's early observation that the immune system can distinguish non-self and promote phagocytosis of the invader, Janeway originally proposed in 1989, that the detection of pathogens is mainly driven by innate immune cells' recognition of pathogen-associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs)[29,30]. Here, the first experimental proof was found in *Drosophila melanogaster*, with the discovery of the toll protein and its role in triggering antifungal responses, soon followed by the description of toll-like receptors (TLRs) in other organisms, including Janeways' work showcasing the role of TLR4 in inducing NF-kB and CD80 co-stimulatory molecule, ultimately indicating for a role in activation of adaptive immune responses[31–33]. Over the years, several other classes of PRRs have been described in addition to the TLRs, namely nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), and absent in melanoma-2 (AIM2)-like receptors (ALRs)[33]. With growing research in the field, came the realization that PRRs recognize common microbe molecular patterns.

Currently, pattern recognition receptors are defined as germline-encoded receptors on innate and NK cells, which can bind and trigger the recognition of common molecules representative of different broad groups of microorganisms. These can include pathogenic or non-pathogenic microorganisms, thus prompting an update in the concept of sensing via PRRs, which is now described to occur through microbe-associated molecular patterns (MAMPs)[33].

Despite being broadly accepted, this model presents major caveats in explaining (1) immune surveillance of evolutionarily distinct microorganisms, to which the host has never been exposed, and (2) tolerance towards non-pathogenetic microbes, such as the host microbiome.

1.1.2.2. The Danger model: sensing of damage-associated molecular patterns (DAMPs)

A complementary model to explain mechanisms of immune surveillance was proposed in 1994 by Matzinger, and is known as “the danger model”[34]. The author argued that rather than identifying what is foreign and non-self, the immune system primarily reacts to damage and stress signals from host tissues through damage-associated molecular patterns (DAMPs).

Thus, according to the danger model, activation of APCs results from sensing danger or alarm signals from host cells injured by exposure to pathogens, toxins, or even mechanical damage. It is important to note that healthy cells undergoing natural death do not release DAMPs. Furthermore, the relevance of PRR-mediated recognition of MAMPs is not disregarded under the danger model but it rather adds that DAMPs are also sensed by these receptors[34,35].

Under the framework of this model, the co-existence of commensal microbial communities, within an organism, that do not elicit immune responses can be explained, as these microbes do not promote injury in host cells. However, it is naïve to consider that the danger model could explain all immune surveillance mechanisms due to its oversimplification of the system, which ultimately fails to explain complex scenarios such as the rise of autoimmunity, asthma, and allergy[36].

1.1.2.3. Effector-triggered immunity (ETI)

Effector-triggered immunity (ETI) was first observed in plants, and later recognized to be a surveillance mechanism also present in other organisms, such as mammals. It stipulates that host cells can recognize pathogen virulence-driven factors. These virulence factors are secreted by the pathogen to manipulate the host in their favor. Detection, under the view of this model of surveillance, is indirect, as host cells do not directly bind the virulence factors but rather detect the modifications or disruptions caused by the pathogen in host cells. Notably, these are distinct from the sensing mechanisms described by the danger model[37].

Following this model, allows the host to distinguish between pathogenic and non-pathogenic agents. Despite being a highly validated model for surveillance in plants, evidence in animals is still scarce, and these surveillance mechanisms fail to explain the detection of pathogens that do not cause physiological perturbations.

Altogether, these models of surveillance attempt to explain how the immune system can detect insults and build an appropriate neutralization and clearance response while simultaneously

being able to recognize self-antigens and microbes that are non-pathogenic and even essential for organismal homeostasis. Separately, each of the models has non-neglectable caveats. However, if we consider that immune surveillance might be dynamic and flexible depending on the context, together they provide good working models to build upon.

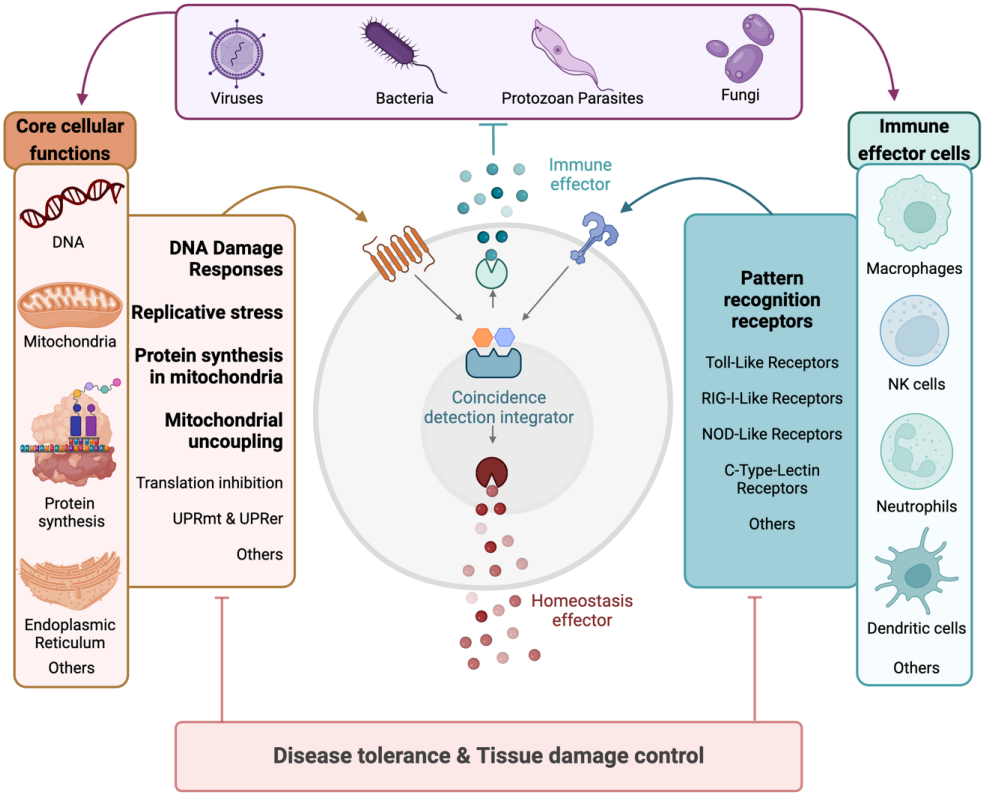

Figure 1. 1 - Models of surveillance mechanisms for the initiation of immune response to an insult.

Illustration created with Biorender (Adapted from [38,39]).

In fact, growing evidence supports a model for immune surveillance wherein pathogen, cellular, and damage signals are perceived by the cell. Herein, the determination of the immune response being triggered results from the integration of all these different signals. Figure 1.1 is a schematic representation of a tentative surveillance model that relies on the synergy of signals perceived by PRRs with cellular stress responses and pathogen-driven mechanisms during infection. This was adapted from the viewpoint of Colaço and Moita (FEBS, 2016) together with the review of Willmann and Moita (Cell Metabolism, 2024), which summarize and reflect on the current studies and evidence within the field of infection and inflammation[38,39]. The concepts of stress responses within homeostasis maintenance and hormesis will be discussed later in this chapter.

1.2. Hematopoiesis in homeostasis and during infection

Organismal homeostasis results from the maintenance of internal equilibrium independently of changing conditions. Herein, immune cell population equilibrium is crucial for proper organismal development, functioning, and health. Maintaining immune cells within the appropriate balance allows for the upkeep of biological processes, efficient responses to insults, as well as of immunological memory, and prevention of the development of aberrant conditions, such as autoimmunity or cancer.

During the embryonic stages and early childhood, immune system development and cell differentiation is a complex and multi-stage process. This is initiated in the yolk sac with the production of primitive erythrocytes and megakaryocytes, followed by the differentiation of early progenitors that will give rise to tissue-resident cells, namely macrophages and mast cells, involved in organ development. Neutrophils, dendritic cells, monocytes, as well as lymphocytes, are primarily differentiated from the fetal bone marrow and establish the innate and adaptive immune system foundation still during embryonic development. Shortly after birth, the immune system's development is changing, with the adaptation of environmental exposures, including the colonization of the gut microbiome[40–42]. Once the development of the immune system has been completed, the maintenance of equilibrium of immune cell populations throughout life and in the response to challenges is managed through immune cell differentiation processes taking place in the bone marrow, described as hematopoiesis.

Adult hematopoiesis is a continuous process occurring in the bone marrow, that is responsible for the production and differentiation of all blood and immune cell populations to maintain the equilibrium. Upon an insult, the hematopoietic process is also responsible for prioritizing the differentiation and maturation of the subset of cells required to deal with the specific challenge. Thus, this is a complex, multi-step process that is tightly regulated as to ensure organismal homeostasis[43]. Due to its importance, it has also been subject to extensive studies.

Within the hematopoietic compartment, in the bone marrow, cells can broadly be classified into three stages: hematopoietic stem cell (HSC) stage, progenitor cell stage, and lineage-committed stage. The HSC stage encompasses long-term HSCs (LT-HSC), and short-term HSCs (ST-HSCs). Herein, LT-HSCs have the greatest self-renewing capacity and provide long-term reconstitution of several bone marrow hematopoietic populations, while ST-HSCs have limited self-renew capacity, when compared to LT-HSCs, but can differentiate into

different lineages. HSCs differentiate into different progenitor types, which have multipotency, albeit already having biases towards specific lineages. In fact, multipotent progenitors (MPPs) have transcriptional profiles that resemble fully committed cells from their respective lineage bias[44,45]. MPPs can produce multiple cell types within their committed lineage[46]. We can subdivide MPPs into megakaryocytic/erythroid- (MPP2), myeloid (MPP3), or lymphoid- (MPP4) biased multipotent progenitors. As a result, these MPPs differentiate into distinct lineage progenitors that will give rise to mature blood and immune cells. The events following the differentiation of the lineage progenitors from MPPs are still highly debated and this remains a controvert topic in certain details.

Figure 1.2 shows the current model of hematopoiesis, constructed considering the most recent findings in the field, described in the works of Yáñez *et al*. (Immunity, 2017), Liu *et al.* (Cell, 2019) and Weinreb *et al*. (Science, 2020), which were nicely reviewed in Swann *et al*. (Nature Reviews Immunology, 2024)[47–50].

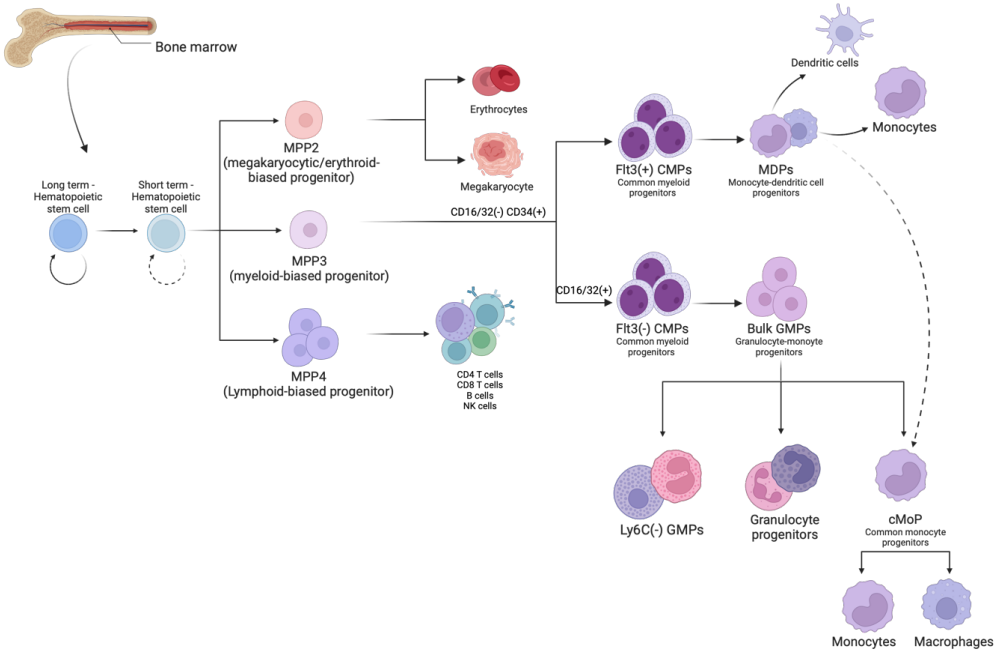

Figure 1. 2 - Hematopoiesis during homeostasis.

Illustration created with Biorender (Adapted from [47–50]).

Despite the ongoing controversial discussion to determine exactly how the differentiation and maturation process during hematopoiesis goes from multipotent progenitors to fully maturated

cells that will leave the bone marrow to enter circulation, it is widely accepted that this is a closely controlled process. Interestingly, it has also been shown that this is a demand-adapted process. Hematopoiesis is not only regulated by the circadian rhythm, as has been described for a long time, but also by external challenges and insults that the host might undergo[50–53]. A growing body of evidence shows that the conventional hematopoietic process can be bypassed during challenges to prioritize differentiation and maturation of specific lineages and cells required for the ongoing challenge[50]. Of particular interest within this work are the changes that occur in the hematopoietic process during infection.

1.2.1. Emergency myelopoiesis and trained immunity

Infections and inflammatory conditions impose a great burden on the host. Herein, a preferential recruitment of immune cells is promoted towards the site of infection. As a result of pathogen neutralization and clearance, many immune cells perish, leading to an imbalance within the immune system. This is strongly reflected in innate immune populations, such as neutrophils, monocytes, and macrophages. These events result in an increased demand for the production of myeloid lineage cells, which in turn induces the reprogramming of the hematopoietic process to prioritize myeloid lineage maturation to the detriment of other cells through a process called emergency myelopoiesis (EM)[50,54,55]. In addition to the immune cell population imbalance, several studies have reported that inflammatory cytokines are also key mediators for the induction of EM[56–59].

The reprogramming that the hematopoietic process suffers during EM can comprise a myriad of changes, depending on the trigger, including metabolic and epigenetic adaptations. This results, most commonly in the activation of HSCs, inducing overproduction of MPP2s and MPP3s, with the possibility of promoting secretory MPP3 populations that promote bypass of the myeloid mechanisms differentiation and promoting self-renewal of granulocyte-monocyte progenitors (GMPs)[50,60–62]. Thus, emergency myelopoiesis is a crucial process for the response of an organism to a challenge. In fact, in pathogen infections, studies show that these epigenetic changes induced during EM in the progenitor cells can become imprinted. This ultimately results in the inheritance of these changes by mature myeloid cells and could be a fundamental process in establishing central trained immunity[60,63,64].

The concept of trained immunity evolved with the first observations that innate immune cells also have some form of immunological memory, demonstrating that they can mount an altered immune response upon re-exposure to a challenge[50,64]. Currently, this is known to be the result

of epigenetic and metabolic imprinting in HSC progenitors, and pilling evidence gathered over the recent years has shown the protective potential of this type of immunity during re-exposure to challenges[63,65]. Thus, it is becoming a promising subject of study for systemic inflammatory conditions and cancer. Despite its importance in organismal responses to challenges, hematopoiesis rewiring and emergency myelopoiesis-induced changes can also have a detrimental effect especially when the conditions inducing these changes persist for long periods of time, such as in the case of chronic inflammation or cancer[50].

1.3. The role of inflammation in infection

Upon detection of an insult and identification of the challenge, immune cells are recruited to the site of challenge initiation, as described previously. During the immune cell response, inflammation is a major player in orchestrating and controlling such responses.

Inflammation is a crucial component of the immune system in response to infection. Physiological processes driven by inflammation promote vasodilation to allow blood flow into the affected area, together with increased permeability of the blood vessels, ultimately allowing for easier migration of immune cells within the organism. Mediators such as histamines and bradykinin are released during inflammation, promoting pathogen isolation and elimination by causing pain and swelling[66,67]. Once the challenge has been dealt with, the inflammatory process also redirects immune efforts to promote damage repair, regeneration, wound healing, and tissue homeostasis. Herein, macrophages play a dual role, being at first involved in pathogen and debris clearing while later releasing cytokines and growth factors that promote tissue repair and regeneration. Additional studies have shown that adaptive immune cells also play a role in tissue repair mechanisms[68,69]. During this stage, these anti-inflammatory mediators suppress inflammation and promote proliferative phase modulating stem cells and progenitors. This ultimately allows for angiogenesis, formation of granulation tissue, and re-epithelialization[69–72].

As in many other biological and immune processes, precise regulation of the inflammation is essential for appropriate responses to insults. Acute inflammation is protective, with short-lived responses from the organism, promoting firstly the control and clearance of the insult, and with the prompt initiation of repair and wound healing mechanisms once the challenge has been overcome. However, when the challenge is not successfully dealt with, or the organism fails to recognize this, chronic inflammation might occur. Chronic inflammation is associated with several autoimmune diseases and complex conditions, such as sepsis. Thus, it is crucial to

disentangle the protective and harmful inflammatory mechanisms and understand how to harness a balanced immune response for the appropriate management of infection and inflammatory diseases[66,67,73].

1.4. Sepsis, a maladaptive host response to an infection

Sepsis is a prime example of a condition resulting from chronic inflammation. It is a life-threatening condition hallmarked by extensive tissue damage and organ dysfunction driven by a maladaptive host response to an infection[74,75]. It is a leading cause of death worldwide, with an incidence of around 47 to 50 million people and an estimate of at least 11 million deaths per year as published in 2020 and shared by Global Sepsis Alliance records for World Sepsis Day[76]. Furthermore, sepsis survivors are, most often, left with life-long consequences, which can include physiological burdens, such as fatigue and weakness, chronic pain and mobility issues, and increased risk of secondary infections; cognitive challenges, such as memory problems and cognitive impairment; and even psychological impacts which can be reflected in depression and anxiety as post-traumatic stress disorder (PTSD)[77]. This ultimately contributes to sepsis being a major cause for concern not only due to its direct impact on patients' individual and familiar lives but also because it represents a great economic burden for health systems[76].

1.4.1. The pathophysiology of sepsis

Sepsis is a multilayered and complex syndrome, encompassing alterations in physiological, pathological, and biochemical parameters triggered by an infection and driven by an aberrant response from the hosts' immune system. This infection can be induced by any invading pathogen and initiated through different infection routes. However, the most predominant cause of sepsis is by bacterial infections, with the most prevalent routes of infection initiation being the lung and the intra-abdominal cavity[78]. Despite the variability in infectious agents and routes of infection that can lead to sepsis, studies show there is a considerable overlap of the transcriptome of blood leukocyte responses shared between different pathogens and infection routes, suggesting a common signature pattern of sepsis pathophysiology[79].

The pathophysiology of sepsis is characterized by an excessive pro-inflammatory response, resulting from the long-term signaling due to an unresolved infection or failure of the host to detect pathogen clearance, and an immunosuppression response, which increases susceptibility to secondary infections. Together, these host responses hinder the capacity to activate tissue repair and regeneration mechanisms required to deal with the damage driven by the pathogen and the hosts' exacerbated response. For a long time, these opposing responses were believed to occur in distinct temporal phases of sepsis. However, recent evidence suggests that excessive inflammation and immunosuppression might overlap and occur in several cycles within an organism while in distinct organs. This also contributes to multi-organ dysfunction syndrome (MODS)[39,80]. The occurrence of septic shock can further complicate the prognosis of sepsis, driving severe hypotension, with the requirement for the use of vasopressors and increased plasma lactate, regardless of volume resuscitation[78].

Thus, the pathophysiology of sepsis results from a series of events and multiple dysregulated mechanisms that can occur in different compartments of organisms. Figure 1.3 shows a comprehensive summary of the several mechanisms leading to sepsis.

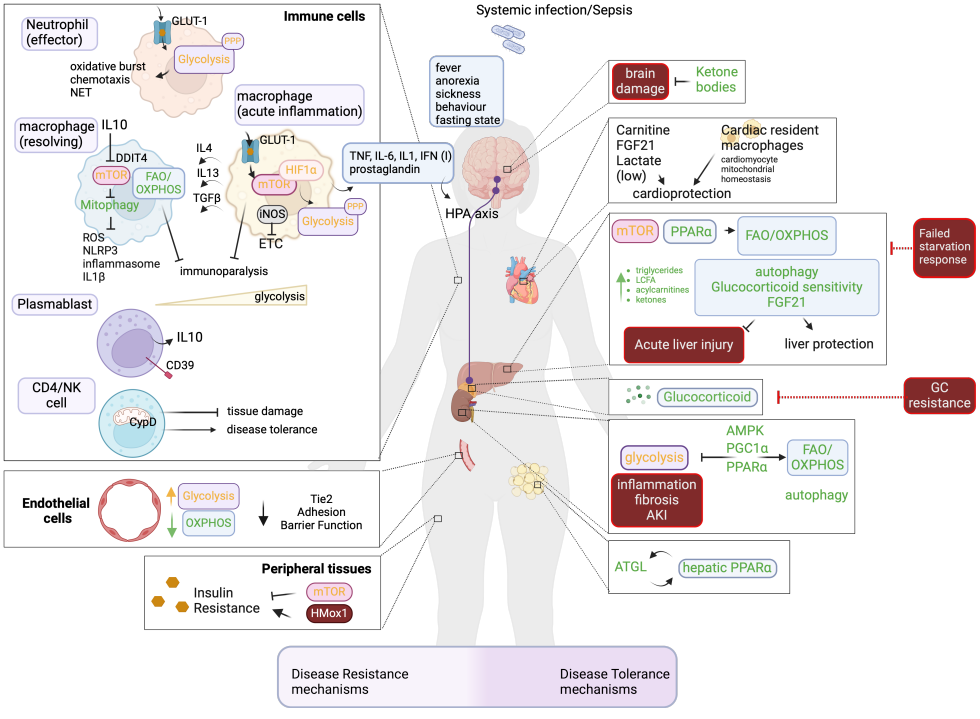

Figure 1. 3 - Dysregulated organismal, tissue and cellular pathways of resistance and tolerance during sepsis.

(Illustration from [39]).

As summarized in the figure above, the dysregulated immune response characteristic of sepsis is driven by multiple deviations from homeostasis, which include cellular, hormonal, bioenergetic, and metabolic alterations. In detail, when a pathogen infects a host, signaling mechanisms, such as described in section 1.1.2. initiate an immune response. This results in the recruitment of innate and adaptive immune cells, as described in 1.1.1.. Upon detection of the challenge, a signaling cascade is activated that promotes NF-κb transcription, leading to the production and release of pro-inflammatory signals, including cytokines such as IL-1, -12, -18, TNFα, different interferons (IFNs), which in turn promote the release IFN-γ, IL-6, -8 while also activating complement and coagulation pathways, this is part of the “cytokine storm”. The excessive inflammatory profile results in failure to control exacerbated responses, which lead to extensive tissue damage, improper pathogen clearance, and ultimately, exhaustion of immune cells, resulting in immune paralysis. Together, these changes in cellular and signaling pathways result in altered energetic demands and metabolic dysfunction[39,81]. In fact, the pro-inflammatory phenotype in sepsis is associated with the promotion of glycolysis-based energetic programs, together with an impaired ability of pyruvate to enter the tricarboxylic acid (TCA) cycle, this ultimately leads to elevated lactate production and metabolic acidosis[82,83]. During the immune suppressive stages, both glycolysis and oxidative phosphorylation (OXPHOS) are compromised, and OXPHOS-reliant biological processes recur to aerobic glycolysis to meet the energetic demands, ultimately resulting in immune paralysis. Due to the high energetic demand during sepsis, the leading metabolic programs are based on catabolic processes, with the breakdown of carbohydrates, lipids, and proteins. The hyperinflammatory stage of sepsis and the high release of inflammatory cytokines impair anabolic processes. This hypercatabolic phenotype is a critical aspect of the development of the metabolic dysfunction associated to sepsis[82–84].

1.4.2. Current diagnosis, therapeutic strategies, and caveats

As a clinical condition predominantly driven by an aberrant immune response of the host, an early and efficient diagnosis of sepsis is crucial to prevent the exacerbation of detrimental phenotypes. Current diagnosis of sepsis results from a combination of clinical assessments, routine laboratory tests, and, when required, imaging exams. Here, clinical assessments are mainly managed through sequential organ failure assessment (SOFA) scoring, laboratory tests aim to identify inflammation (scores of inflammation (SIRS)) and organ dysfunction. These encompass blood cultures to identify potential infectious pathogens, blood count, and

leukocyte profiling, blood clotting analysis, liver and kidney function enzyme assessment, oxygen levels, and electrolyte imbalances. When the source of infection is not clear, imaging exams can be performed to help clarify the condition. These might include X-rays, ultrasounds, and CT scans[74,85,86].

Once a patient is suspected to have sepsis, the therapeutic strategy employed depends on the patient's clinical condition. The most common mode of action is to initiate antimicrobial therapies that promote pathogen clearance. Here, the appropriate identification of the pathogen allows for proper monitoring of the efficiency of containment of the infectious agent and correspondent de-escalation of the targeted therapy. In parallel, to support adequate blood volume and circulation, resuscitation strategies consisting of the administration of intravenous fluids, namely crystalloids, are employed. In more severe cases, such as septic shock and respiratory failure, vasoactive mediators, such as norepinephrine can be used to maintain blood pressure and organ perfusion, and supportive care can be provided by mechanical ventilation. Additionally, heparin administration can be used to prevent venous thromboembolism and patients should be monitored to control glycemia levels, to prevent the development of hyper- or hypo-glycemic conditions, which are associated with worse outcomes and even increased mortality in sepsis[75,86].

Despite being a highly researched topic, diagnosis and treatment strategies for sepsis are far from ideal. The complicated and non-specific nature of symptoms associated with sepsis, together with the lack of gold standard tests to identify the condition early on, impair an efficient diagnosis of patients. Furthermore, currently, available clinical strategies for treatment fail to tackle the metabolic dysfunction hallmarking sepsis and fall short of restoring homeostatic balances within the immune system. Thus, it is of great interest for sepsis and many other infectious and inflammatory conditions to understand how mechanisms of resistance and tolerance are balanced and how, in dysregulated conditions, homeostasis can be restored.

1.5. Finding the balance: disease tolerance and resistance mechanisms

An organism employs two main defense strategies during infections: disease resistance and tolerance. Resistance strategies include all mechanisms that aim to kill and clear the pathogen. These include activation of innate and adaptive immune response following sensing by surveillance mechanisms, together with immune signaling of cytokines and pro-

inflammatory mediators. On the other hand, the interest in disease tolerance mechanisms has grown over the last few years within the field of sepsis. Disease tolerance was first observed and described in plants and has, over the years, been shown to be a highly evolutionarily conserved mechanism across species[87,88]. It includes all mechanisms that do not have any direct impact on the pathogen but rather focus on minimizing the infection-driven damage, caused directly by the pathogen or resulting from the exacerbated immune resistance response of the host. Herein, metabolic reprogramming, tissue repair processes, and modulation and control of immune responses to prevent excessive inflammation are crucial for host health[89–91]. Importantly, the concepts of immunological tolerance and disease tolerance are distinct and refer to two separate functions of the immune system, where the first explains how immune cells can recognize and tolerate self-antigens, and the latter refers exclusively to responses during infection and disease[92].

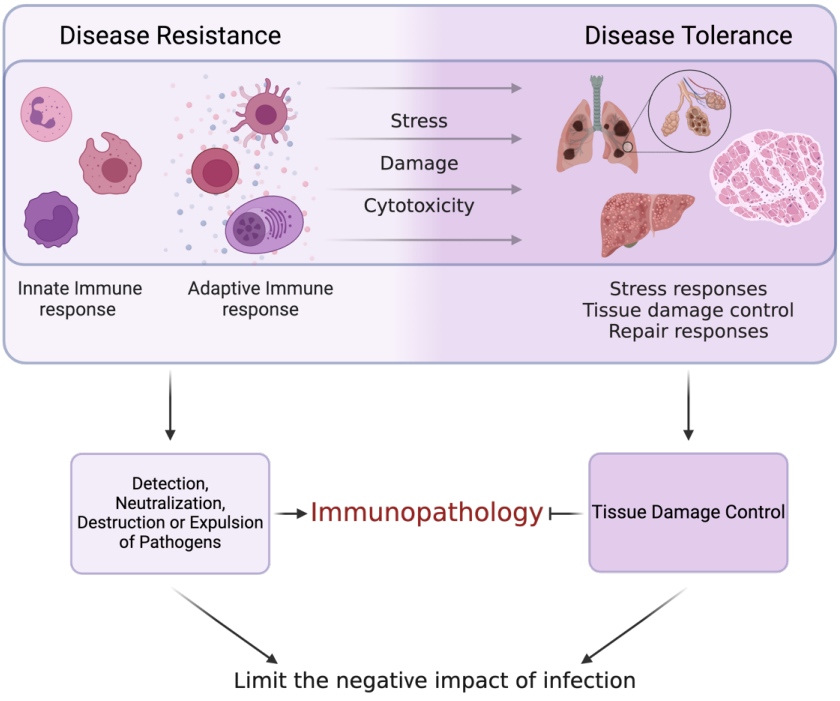

Figure 1. 4 – Disease resistance and tolerance strategies during infection and inflammation.

Illustration created with Biorender (Adapted from [39])

An appropriate balance between resistance and tolerance is required to establish an effective immune response during a challenge. This allows the host to promote pathogen clearance responses while simultaneously preventing overactivation of the immune system and

consequent exacerbation of tissue damage. In line with this, imbalances in resistance and tolerance programs are at the basis of various pathologies such as COVID-19, primary immunodeficiency disorders, autoimmune diseases, and chronic inflammatory conditions, including sepsis[93–95].

A growing body of evidence suggests that modulating the immune system could have protective effects on the resolution of sepsis. Herein, direct immunomodulatory drugs have been tested for the promotion of pro-inflammatory mechanisms (such as administration of GM-CSF, Tα1, IFN-γ, amongst others) during the immune suppression phenotypes or immune regulatory drugs for control of exacerbated immune responses during the hyperinflammatory responses (namely blocking antibodies for IL-1ra, TNFα, IL3, and TLR4, or other therapeutic strategies such as the use of glucocorticoids)[95]. Although, in certain cases, these strategies have yielded improvements, they are highly dependent on the clinical condition of the patients and ultimately require a deep understanding of individual clinical profiles for choosing the adequate immunomodulatory treatment.

A promising emerging approach for the modulation of host responses' that has been gaining more traction is harnessing stress responses[96]. Thus, understanding how resistance and tolerance mechanisms contribute to infection, and how stress mechanisms can lead to the activation of these specific programs can potentially improve the current understanding and development of treatment strategies for conditions resulting from homeostatic disruptions[90].

1.6. Stress responses in the maintenance of organismal homeostasis

The idea of "stability of the internal milieu" was first suggested by Claude Bernard in 1878 but was only coined later by Walter B. Cannon in 1929. In his work, Cannon described the concept of homeostasis as "the coordinated physiological reactions which maintain most of the steady states in the body". The author further acknowledged that the maintenance of homeostasis is reliant on the existence of passive and active mechanisms for the detection of deviations within an organism[97,98].

The mechanisms that help maintain organismal homeostasis have since been of great interest to the physiology field. This has allowed us to understand that multicellular organisms have evolved tightly regulated mechanisms to control their temperature, pH, glucose, and ion levels, cell numbers and proportions, within the optimal range for appropriate biological function.

Additionally, maintenance and regulation of homeostasis relies on surveillance mechanisms, which are responsible for detecting deviations from balance, and promoting compensatory mechanisms to respond to the changes, ultimately leading to the restoration of homeostasis[98,99]. Within the compensatory mechanisms, stress responses are vital for adapting the organisms to a challenge[100].

Stress responses can encompass cellular, biochemical, and even physiological reactions from an organism to an insult. The intracellular consequences of stress responses have been subject to extensive study and are described in the literature to a modest degree.

These cellular stress responses include DNA damage and unfolded protein responses (UPRs), autophagy, and mitochondrial stress signaling. In more detail, DNA damage responses can be activated by damage caused by radiation, oxidative stress, or even specific drugs, such as anthracyclines. These lead to activation of repair pathways and, if necessary, cell cycle arrest. Activation of DNA damage responses ultimately promotes restoration of homeostasis by preventing mutations and maintaining genomic integrity. Protein quality is essential for cellular function. Accumulation of misfolded proteins in the endoplasmic reticulum leads to the activation of unfolded protein responses (UPRs). These aim at promoting protein folding capacity and degradation of misfolded proteins, ultimately protecting cells from stress-induced apoptosis. Similarly, autophagy is a process responsible for degrading and recycling damaged cellular components. These can include proteins and even whole organelles and can be activated due to nutrient starvation or other stressors. This ultimately allows the cells to improve their nutrient availability while, in parallel, removing damaged and possibly toxic structures. Mitochondrial stress signaling can have a myriad of impacts due to numerous biological processes in which mitochondrial function is involved. Mitochondrial stress can influence the bioenergetic and metabolic functioning of the cells, but it has also been reported to be involved in UPRs and autophagy[100].

The above-described roles of stress responses in maintaining homeostasis have been extensively investigated in simple model organisms, such as *C. elegans,* and studies have proven these are conserved mechanisms in mammals[101]. The systemic implications of stress responses are poorly understood. Nonetheless, a growing body of evidence within this field suggests that harnessing stress responses could have potential applications in restoring homeostasis in complex conditions. In fact, research suggests that stress responses are a fundamental tool for hormesis.

1.6.1. The role of hormesis in infection

The term hormesis was first described in 1943 by Southam and Ehrlich, but evidence for these processes has been around since 120 B.C., when King Mithridates VI of Pontus, fearing his assassination by poison, decided to regularly ingest small portions of a mixture of several toxic substances to build up resistance and protect himself[102,103].

Hormesis describes the biological phenomenon by which cells or organisms better adapt to a challenge after exposure to a lower dose of a toxic substance or stress[104]. It encompasses a biphasic response, wherein low doses of stressors can stimulate a beneficial and protective effect against secondary challenges, while high-dose exposure to the same stressors can have hindering, toxic or even lethal effects. Thus, it is a highly dose-dependent process[105].

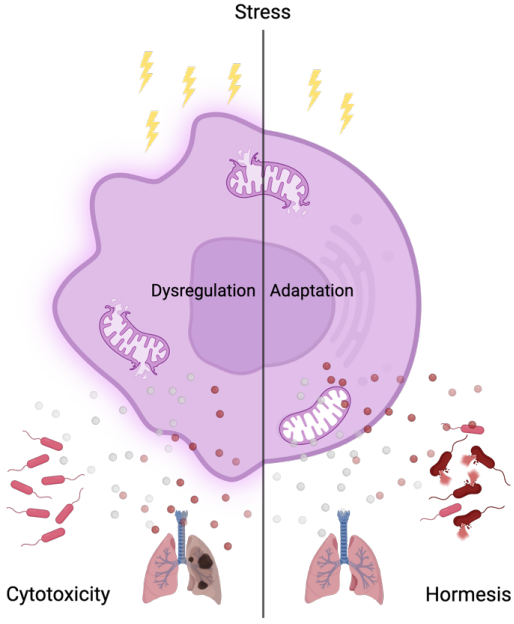

Figure 1. 5 - The concept of hormesis: dose-dependent cellular responses to stress.

Illustration created with Biorender (Adapted from [106])

Since its conception, many research studies have shown the promising roles of hormesis, from improving adaptability to lifespan expansion. From the results first described by Southam and Ehrlich, showing that low-dose exposure to red cider tree extracts could enhance fungal metabolism. To bacterial improvement of resistance, and promotion of biofilm by low-dose antibiotic exposure adaptation, to improving yeast resistance to hydrogen peroxide by subsequential low-dose exposure to the compound[102,107–109]. The many studies demonstrating the lifespan expansion potential of hormesis, have been nicely reviewed by Calabrese *et al.*

(Ageing Research Reviews, 2024)[110]. The roles of hormesis in physiology are broad and appear to be, to some extent, evolutionarily conserved.

The potential of using hormesis to protect against infection and inflammation has become even more important with the increasing evidence that this could be used to modulate the host immune response, with consequences in cell migration, function, and cytokine release[111.]

For one, studies have shown that monocytes and other innate immune cells can be pre-conditioned by low exposure to stressors to improve clearance responses to subsequential infections by epigenetic and metabolic reprogramming of cells, an observation that has since been classified as trained immunity[65,112–115]. On the other hand, growing evidence shows the potential of stress responses to harness disease tolerance mechanisms during chronic inflammation. From the simplest exemplification, that moderate exercise can have anti-inflammatory effects and provide health benefits, to studies describing hormesis conferred protection of inflammation-driven damage in different conditions[109,116]. To list some, one study has shown that harnessing integrated stress responses can protect during the neurogenerative condition of multiple sclerosis by maintaining proteostasis, which results in an effective protection of oligodendrocytes and myelin from inflammation[117]. Low-dose exposure to nitric oxide has been shown to have anti-inflammatory properties and contribute to cardiovascular health and adaptation to intermitted hypoxia[118]. Within the field of autoimmunity, low-dose exposure to radiation has been proposed as a treatment strategy to ameliorate disease-induced damage[119]. Even within the cancer research field, promoting hormesis has gathered some compelling evidence for new therapeutic approaches[120,121].

Specifically in the field of sepsis, mechanisms based on DNA damage responses, and mitochondrial stress have been described by several authors to harness protective programs. Indeed, our lab has been at the forefront of dissecting how mild perturbation in core cellular function could protect during infection and inflammation, using mice models of sepsis. Our work, together with evidence from other independent researchers, has shown that anthracyclines, particularly epirubicin, confer protection from severe sepsis by activation of DNA damage responses and autophagy pathways in the lung, and that this protective phenotype is dependent on Sphingosine 1-phosphate signalling[122,123]. Most recently, we have shown that mild mitochondrial stress elicits protective disease tolerance mechanisms in bacterial sepsis. In this study, mitochondrial stress improved survival and reduced tissue damage scores of severely septic mice, independently of pathogen burden[124]. In line with our findings, the work of Mottis *et al*. (Journal of Clinical Investigation, 2022) has also shown mitochondrial stress-mediated disease tolerance protective mechanisms in an influenza infection model[125].

Although studies show evidence for perturbation of different core cellular functions having the capacity to elicit protection, and hormesis, the mitochondria is a target of particular interest in the field of infection and inflammation due to its broad cellular and physiological roles within an organism and its sticking and significant connections to hormesis. In line with this, the concept of mitohormesis was created to encompass all mechanisms by which mitochondrial stress can promote the restoration of organismal homeostasis[126].

1.7. The organismal roles of the mitochondria

Mitohormesis, stems from the concept of hormesis, and describes adaptive responses underlying protective mechanisms that can improve health and increase lifespan triggered by mild mitochondrial stress. Albeit the concept of hormesis, encompasses all stress responses leading to adaptation phenotypes, the ample number of physiological processes in which mitochondria are involved, and the myriads of functions they fulfill in health and disease, led to the conception of the field of mitohormesis[103].

Mitochondria are notoriously known for their role as “the powerhouse of the cell”. Ever since the first evidence linking mitochondria with bioenergetics of a cell by Kingsbury in 1912, the field of mitochondria research has massively grown[127,128]. As a result, an increasing body of evidence has been showing that the functional impact of mitochondria within an organism surpasses greatly their energy production role and, in turn, its function highly impacts health and disease.

Mitochondria are membrane-bound organelles that can be found in the cytoplasm of most eukaryotic cells. Unlike other organelles, mitochondria have their own genome (mtDNA), which is circular DNA, composed of 37 genes that encode two ribosomal RNAs, 22 transfer RNAs, and the respiratory subunits of the electron transport chain (ETC). Despite having their DNA, mitochondria rely heavily on nuclear DNA. In fact, nuclear-encoded mitochondrial proteins represent the vast majority of proteins required for mitochondrial function within a cell and the organism[129].

The presence of mitochondria within cells is explained by a theory widely accepted within the scientific community, known as the Endosymbiotic theory[130]. This theory proposes that mitochondria originated from bacteria that were engulfed by an ancient archaeal host cell around 1.6 to 2.2 billion years ago. This does not only explain the many resembling features between mitochondria and bacteria (such as the structure of their DNA) but also represents a

crucial event for the evolution of eukaryotes[130]. Interestingly, the existence of cell-free mitochondria was reported in a study by Dache *et al*. (FASEB J., 2020) with results showing that these are respiratory competent and the suggestion that they could be involved in cell-to-cell communication[131]. The findings regarding the functional respiratory capacity of the cell-free mitochondria have, however been a target of debate within the field, with no reliable conclusion to be drawn from as of this moment[132]. Regardless, the dependency of cells in mitochondrial processes is clear and reflected in several biological processes.

1.7.1. Bioenergetic processes

As referenced before, the most known and studied function of mitochondria is to provide energy to maintain biological processes within an organism. Mitochondria break down nutrients into adenosine triphosphate (ATP) to produce energy. This is achieved, the majority of times, by oxidative phosphorylation (OXPHOS). For this, acetyl-CoA is broken down from carbohydrates, fats, or proteins in the mitochondrial matrix, and this leads to the production of NADH and FADH2, which carry electrons to the electron transport chain (ETC). The ETC is composed of four protein complexes (I to IV), ubiquinone, and cytochrome C (which are electron carriers). Here, the electrons from NADH and FADH2 pass through the complexes and create an electron flow, while a proton pump creates a proton gradient from the matrix to the intermembrane space. Lastly, complex V of the ETC, also known as ATP synthase, converts ADP into ATP using the proton gradient by a process called chemiosmosis[133]. In tissues with high energy requirements or during nutrient starvation, to fulfill the energetic demand, fatty acid oxidation (FAO) occurs within the mitochondria of cells, through the process of β-oxidation, by which fatty acids are broken down to serve as sources for ATP production[128]. While OXPHOS is the most efficient energy production process of cells, it also leads to the release of byproducts, namely reactive oxygen species (ROS), which in higher doses can become harmful and modulate cellular signaling[134].

Cells can also use glycolysis for energy production. This consists of a metabolic pathway that breaks down glucose into pyruvate, producing ATP and NADH. It can occur in aerobic or anaerobic conditions, which result in different end-product fates: in aerobic glycolysis, pyruvate enters the mitochondria for further oxidation, while in anaerobic glycolysis, pyruvate is converted into lactate and ethanol[135]. Thus, despite not occurring directly in the mitochondria, the glycolysis process is influenced and releases products that will directly impact mitochondrial respiration. Regardless of the lower efficiency of glycolysis, it is considerably faster than OXPHOS. As a result of this, in conditions in which cells require

immediate or fast energy availability, the preferential energetic program might be adapted to meet the demands[128].

1.7.2. Signaling and cell death

Besides their well-described role in maintaining the cell's bioenergetics, mitochondria are also fundamental signaling hubs within the cell and for cell-to-cell communication. In fact, the Nobel Prize in Physiology or Medicine from 1953 was attributed to Hans Krebs for his work describing the role of the citric acid cycle, most commonly referred to as the tricarboxylic acid (TCA) cycle, in energy production and generating intermediate metabolites[136,137].

Mitochondrial signaling pathways have been subject to extensive study over the years. From the early findings describing the role of cytochrome C release by the mitochondria in the initiation of cell death processes through apoptosis, the understanding of the depth and complexity of the involvement of mitochondrial signaling in biological processes has increased greatly[137–139]. In addition to the involvement in cell death, mitochondrial signaling has been implicated since early studies with the induction of hypoxic genes through ROS signaling, control of calcium signaling, by regulation of cytosolic levels of Ca^{2+}, subtract phosphorylation rates, by promoting the formation of protein-kinase complexes in the mitochondrial outer membrane and even induction of stress responses, with the earliest evidence being the release of mitochondrial-specific heat shock proteins to promote cytosolic calcium signaling. More recent studies have brought evidence to light for additional pathways, which include metabolite-, mitochondrial dynamic (fission and fusion)-, DAMP-, and mitochondrial-derived peptide-mediated signaling. In addition to the intracellular signaling roles of the mitochondria, it has already been described that these organelles can be transferred between cells, with results showing a biological impact on wound healing and cardiovascular function[140–143].

Together with studies that have shown mitochondria can act as a sensor and modulator of cellular dynamics and environmental changes, the phenomenon of mitochondrial retrograde signaling, consisting of the mechanisms by which the mitochondria are also involved in communicating their own status, supports the importance of mitochondrial fitness in modulating nuclear gene expression[144]. In addition to the functional ability to meet bioenergetic requirements of the cellular process, mitochondrial dynamics and motility, including the processes of fusion, fission, intracellular trafficking, and interaction with other organelles, such as the endoplasmic reticulum, can influence gene expression and signaling. Figure 1.6, illustrates the functions and dynamics of mitochondria that are involved in maintaining cellular homeostasis.

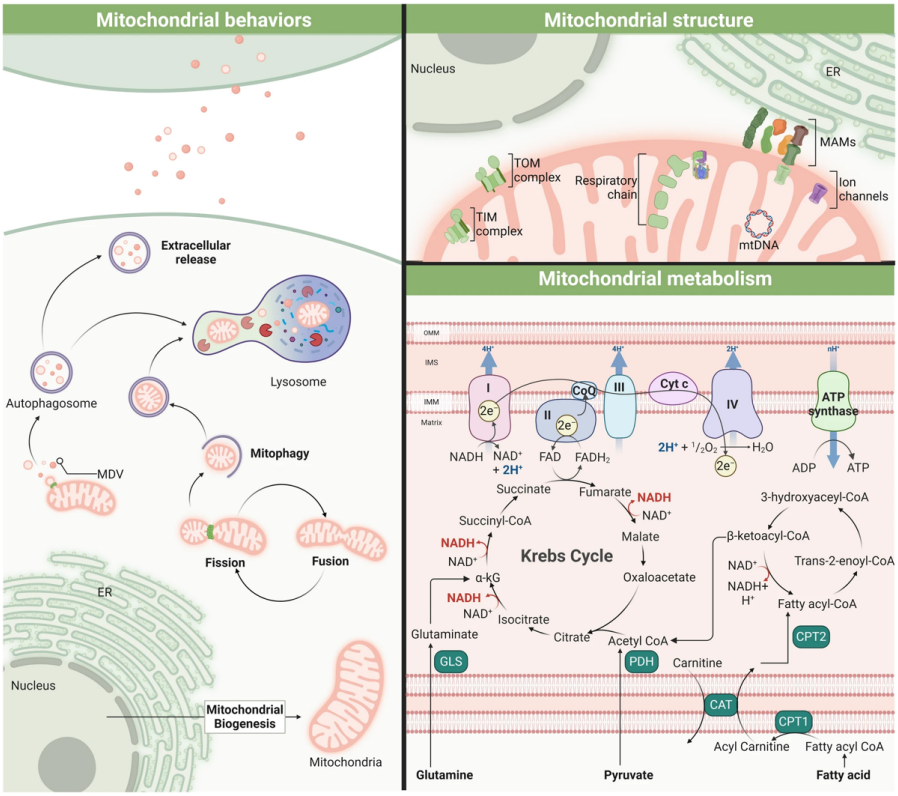

Figure 1. 6 - Mitochondrial behaviors, structure and metabolism involved in the maintenance of cellular homeostasis by contributing to cellular dynamics, bioenergetics and signaling processes.

(Illustration from [145])

Altogether, there is solid data in the literature to support the crucial role of mitochondria in an organism that surpasses the bioenergetic properties of these organelles and that there is a significant contribution of mitochondria in the orchestration of intracellular pathways and extracellular communication. Not surprisingly, the disturbance of mitochondrial homeostasis

has been shown to have extensive organismal repercussions, which can encompass neurological, metabolic, cancer, age-related and inflammatory conditions[139,145].

1.7.3. Targeting the mitochondrial ETC

Over the years, targeting the mitochondrial electron transport chain pathways by drugs or toxins has become of particular interest. This followed the first observations that many classes of pharmaceuticals used to treat different conditions had off-target effects on mitochondrial functions, ultimately leading to major side effects of the treatments[146,147]. The main mechanisms of mitochondrial toxicity described so far, include direct inhibition of ETC complexes, uncoupling of OXPHOS and consequent disruption of proton gradient necessary for ATP production, induction of oxidative stress resulting in increased ROS and damage to ETC components, and interference with mtDNA interfering with ETC protein synthesis. Altogether, the consequences of chronic exposure resulting in mitochondrial toxicity have been associated with pathological conditions that lead to liver injury, muscle weakness and myopathy, neuropathy, lactic acidosis, and lipodystrophy[147–150].

Amongst the major drug classes reported to induce mitochondrial toxicity, examples include chemotherapeutic agents, antipsychotics, antiviral and antibiotic treatments, and even antidiabetic drugs, such as phenformin and metformin, with the last one representing by far the most extensively studied drug[147,148,151–154]. In more detail, besides their canonically reported role in gluconeogenesis suppression of biguanides, this particular class of antidiabetic drugs has been reported to have impacts on mitochondrial function including inhibition of complex I, uncoupling, iron overload, and TCA cycle inhibition[154–156]. These pleiotropic effects of metformin and phenformin ultimately led to their association with improved anti-cancer, infection, and inflammatory responses[157–159].

The increased awareness of the broad range of impacts of mitochondrial toxicity has highlighted the importance of a more thorough drug safety assessment and even led to the creation of a mitochondrial toxicity database[160]. On the other hand, it has also opened new avenues of research, in which the detailed dissection and understanding of the systemic impact of mitochondrial targeting could be explored for disease treatments.

1.7.4. The impact of mitochondrial fitness on immune cells

Considering the broad range of biological functions in which mitochondria are involved, understanding the impact of inducing mitochondrial stress and finding mitohormesis mechanisms has become extremely interesting within different fields of biology and biomedicine[103]. This is of particular interest in infection and inflammation, considering the close evolutionary ties that mitochondria and bacteria share. Especially considering that bacteria are one of the major causes of infection leading to sepsis, as well as the metabolic and energetic dysregulation that hallmarks extreme infections and chronic inflammatory conditions, such as sepsis[161]. Thus, research studies have been accumulating evidence for the potential of targeting mitohormesis as a tool for therapeutic approaches.

Of key importance for the field, was the study published by Durieux *et al*. (Cell, 2011), showing that manipulations of the ETC function modulate aging in a cell-non-autonomous fashion, with evidence that the mitochondrial stress of one tissue can be perceived within the whole organism and induce organismal changes[162]. The authors justified this phenomenon by mitochondrial signaling through soluble mediators, designated “mitokines”. As a result of this, several other research groups have reported the identification of other mitokines, for example neuronal peptide FLP2, fibroblast growth factor 21 (FGF21), growth differentiation factor 15 (GDF15), and many mitochondrial DNA-encoded peptides[163–165]. Interestingly, literature describing the biological relevance of these signaling mediators seems controversial, indicating different roles depending on whether there is acute or chronic exposure, which is in fact, well in line with the concept of hormesis[166].

Despite the increasing amount of research being published in the field, the impact of mitochondrial stress in different immune cells remains in high need of more comprehensive studies. Currently, in addition to the canonical role in cellular signaling, it is broadly described in the literature that mitochondrial stress is involved in immunometabolism, with an essential role in the metabolic reprogramming of immune cells to support effector function[167,168].

On the one hand, innate immune cells’ bioenergetic programs have been subject to characterization, and it is well-defined that their reliance on OXPHOS or glycolysis for energy production is a key regulator of their effector profile. For example, Freemerman *et al.* (JBC, 2014), showed that macrophages can be metabolically reprogrammed to a pro-inflammatory phenotype by the overexpression of glucose transporter 1 (GLUT1), an observation that was later validated by other studies[169,170]. As for the mitochondrial role in innate immune cell signaling, it is well described that mitochondrial stress triggers the release of specific DAMPs, such as mtDNA, ROS, and ATP, amongst others, and in turn, these DAMPs will activate PRRs from innate immune cells initiating inflammatory responses[171–173]. In line with this, studies have shown that mitochondrial ROS and the release of oxidized mtDNA during the NETosis process

have crucial roles in promoting inflammation during autoimmune conditions and cancer progression[174,175]. Similar results have been described in innate immune system activation with signaling through double-stranded mitochondrial RNA release, double-stranded mtDNA breaks, and RNA-DNA hybrids occurrence[176–178].

Interestingly, there are also growing numbers of studies reporting the role of mitochondrial stress in inducing mitohormesis mechanisms, which promote tolerance and protection from exacerbated inflammation. Herein, studies have reported that mitohormesis via oxidative and electrophilic mitochondrial stress induces tolerance and control of the inflammatory response to LPS-stimulated macrophages *in vitro* and *in vivo*, reducing inflammation-driven damage[179]. Other studies have demonstrated that targeting the mitophagy pathway, the process by which damaged or depolarized mitochondria are removed from the cells, can promote different responses. Defective removal has been shown to be associated with hyperactivation and inflammation, by NLPR3 inflammasome activation[180,181]. However, other studies have shown that inhibition of mitophagy could suppress type-I interferon signaling[182,183]. These discrepancies in the biological effect of mitophagy suppression were justified because the target cells in the studies resort to different surveillance pathways[182].

Many studies have also described the importance of mitochondrial function and signaling in adaptive immune cells[184]. The adaptive immune responses can be affected by mitochondrial stress indirectly, as a result of the modulation of innate immune cells by the mitohormetic stress, or directly with consequences in bioenergetic and immunometabolism of the cell, which ultimately can be reflected in proliferation, fate decisions and effector functions of these cells. Results in this field have allowed us to understand that adaptive immune cells heavily rely on different bioenergetic programs depending on their functional requirement, as exemplified by the reports showing that activated CD4 T helper cells switch their main bioenergetic source from mitochondrial respiration through OXPHOS to aerobic glycolysis during proliferation, differentiation, and effector function, in parallel, T regulatory cells resort in the large majority to OXPHOS programs, except during migration[185–187]. In fact, experiments using genetic manipulation to impair glucose uptake have proven that failure to switch to glycolysis impairs T-cell proliferation and activation[185]. Mitochondrial damage signaling molecules also have an impact on lymphocytes. Some key examples of this include studies that have reported ROS signaling is important for antigen-specific T cell activation and expansion events, that the metabolic reprogramming required for memory CD8 T cytotoxic cells is dependent on mitochondria-endoplasmic reticulum contact sites and that lymphocytes, especially B cells, use the secretion of mtDNA webs (similarly to the NETosis process observed in neutrophils) when challenged with CpG and non-CpG oligodeoxynucleotides of class C[188–190].

Natural killer cells are categorized as innate immune cells of lymphoid origin. As a result of their biological ancestry and effector functions, these cells share many commonalities with both immune arms. However, they also have distinctive features, from both innate and adaptive cells that separate them from any. Because of this, precise literature showing the impact of mitochondrial stress in NK cells is scarce and incomplete, and the understanding of the role of mitohormesis in these cells requires further research.

In the recent years, some studies have begun to address the role of mitochondrial stress in NK cells. What has been reported thus far shows that in terms of the bioenergetic role of the mitochondria, NK cells resort to both glycolysis and OXPHOS to meet their energetic demands. Interestingly, the work of Wang *et al.* (Frontiers in Immunology, 2020) showed that inhibition of glycolysis (but not OXPHOS) decreased NK cell-mediated killing and the degranulation capacity of the cells during NK cell receptor-mediated activation[191]. In addition, during the last year, studies have reported that mitochondrial stress leads to a decrease in viability and altered metabolism, which results in impaired activation, proliferation, and cytotoxic capacity, with indications that exposure to mitochondrial stress might induce NK cell exhaustion[192–194].

Taken together, the evidence being gathered supports the crucial role of mitochondria in the effector function, fate-decision, and viability of all subsets of immune cells. The functional responsibilities of the mitochondria within immune cells surpass their role in bioenergetic production, with a strong component relating to mitochondrial signaling. The elucidation of detailed mitochondrial mechanisms and changes leading to the orchestration of the various immune responses in different cells has, however, proven to be a subject of extreme complexity. This is not only because of the broad spectrum of functions the mitochondria fulfill within a cell but also because of the different core bioenergetic requirements, surveillance, and response mechanisms employed by default by distinct immune cells. Currently, it is well established that mitochondrial stress can promote organismal changes and that if the stress is well dosed, mitohormesis can be triggered. Considering the growing literature, supporting the protective capacity of mitohormesis, it becomes of particular interest to understand how a specific stress in the mitochondria can induce organismal protection. To answer this question, scientific studies should focus on systemically and comprehensively characterizing the type of mitochondrial stress being induced and the phenotypical and dynamic organismal changes, with particular interest in the different immune cell populations. This could ultimately help elucidate what mechanisms lay in the promotion of hormesis and provide insights into how

one can promote proper pathogen clearance while still permitting inflammation resolution and avoiding extensive tissue damage due to aberrant immune responses.

1.8. Thesis aims and outline

A growing body of evidence suggests that the ability of stress responses to promote restoration of homeostasis after an insult is a highly evolutionarily conserved mechanism. However, the systemic study of the impact of these stress responses in higher organisms, such as mammals, is still scarce and incomplete.

Following the observations, (1) that mild mitochondrial stress protects mice from sepsis through disease tolerance mechanisms[124], and the studies describing (2) how mitochondrial perturbations can elicit innate immunity-related mechanisms in *C. elegans*[195,196] (further supported by the most recent findings in *D. melanogaster*[197]), we set out to understand the systemic impact of mild mitochondrial stress in tolerance and resistance responses during infection, by using a murine model of mild sepsis. With this work, we aimed at:

1. Studying the ability of mild mitochondrial stress to induce disease tolerance and resistance mechanisms;
2. Understanding the key immune dynamic changes elicited by mitochondrial stress during infection;
3. Dissecting the possible mechanisms by which immune cell dynamics contribute to tolerance or resistance mechanisms.

Throughout this work, several aspects of the protective mechanisms elicited by mild mitochondrial stress will be subject to discussion:

In ***Chapter 2,*** we tested the ability of mild mitochondrial stress to elicit tolerance and resistance mechanisms in a mild murine model of sepsis. We found that phenformin can protect mild septic mice by promoting tolerance mechanisms at early time points of infection, reducing tissue damage without imposing any effect on the pathogen. While, during later time points, allowing for a switch towards resistance mechanisms, which in turn support a more efficient pathogen neutralization and clearance capacity. These effects were found to be independent of possible direct effects of the treatment on the pathogen and seem to be distinct from the gluconeogenetic suppressive capacity of phenformin, indicating that they are a result of the strong complex I inhibition of the mitochondrial ETC provided by phenformin.

Chapter 3 describes the characterization of systemic immune dynamic changes during infection elicited by our treatment. This has allowed us to identify core immune cell populations associated with tolerance and resistance mechanisms. We show that phenformin treatment promotes control of lymphocyte and NK cell populations infection-driven activation, demonstrated by the reduction of the early activation marker CD69 expression on these cell subsets. On the other hand, treatment promotes preferential recruitment of neutrophils and monocytes to the infection initiation site, the peritoneal cavity, and controls exacerbated influx of these cells to the lung. Interestingly, phenformin triggers a macrophage disturbance of homeostasis reaction in the peritoneal cavity in non-infection conditions.

Chapter 4 focuses on dissecting the biological impact of the immune dynamic changes reported in Chapter 3. Thus far, we have not been able to validate the role of CD69 downregulation on lymphocytes and NK cells in tolerance mechanisms and reduction of tissue damage. More work is required to elucidate the relevance of these changes in protecting mice from sepsis. Nonetheless, this work uncovered that phenformin treatment modulates hematopoiesis, promoting emergency myelopoiesis and more rapid differentiation of myeloid lineage immune cells such as neutrophils and macrophages. This translated to earlier recruitment of these cells to the peritoneal cavity during infection, which we speculate provides protection by allowing for prompt responses by the neutrophils and faster replenishment of tissue-resident macrophages during infection, a core population in peritoneal bacterial clearance. Together, these data could indicate a possible role in phenformin in promoting trained immunity, which requires further investigation.

In ***Chapter 5,*** the key findings described throughout the chapters of this work are discussed in an integrated fashion, including possible biological relevance and speculations considering data published by other authors. Additionally, future perspectives and possible experimental approaches are described for a more mechanistic elucidation of the results presented in this work.

Appendices 1[198] ***and 2***[199] are commentary publications produced during the course of this PhD work, on relevant scientific studies that, in line with previous work of our lab, have highlighted the importance of disease tolerance mechanisms in infection and inflammation.

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Chapter 2

Phenformin protects against bacterial sepsis by activating host-dependent disease tolerance and resistance mechanisms

Author contributions

Kátia Jesus was responsible for planning, executing and running analysis scripts on data from all experiments in this chapter. André Barros and Elsa Seixas provided assistance in the *in vivo* work. André Barros developed the scripts for statistical analysis. Luís Ferreira Moita supervised the project and reviewed this chapter.

2.1. Abstract

Sepsis is one of the leading causes of death worldwide. Besides the alarming mortality, sepsis survivors are also left with life-long consequences that impose great personal and economic burdens. This is mainly due to the extensive tissue damage driven by sepsis that ultimately leads to organ failure. This has inspired the sepsis research field to shift from exclusively targeting the pathogen (the origin of the inflammatory condition) to studying host-modulating-based therapeutic strategies.

Growing evidence supports the potential of stress responses in harnessing disease tolerance mechanisms. These promote tissue protection programs, providing a tool to avoid extensive organ damage. However, the balance between resistance and tolerance programs must be maintained to treat an inflammatory condition adequately. Thus, it becomes urgent to understand the impact of triggering stress responses and promoting disease tolerance on the resistance mechanisms.

Here, we used a bacterial peritonitis mouse model of sepsis to test the protective effect of phenformin-induced mild mitochondrial stress in disease tolerance and resistance mechanisms. In line with our previous results, we found that mild mitochondrial stress protected septic mice in an initial stage by promoting disease tolerance mechanisms, with reduced tissue damage and no changes in pathogen burden. Interestingly, at a later stage, phenformin-treated mice showed improved clearing capacity compared to control animals, supporting the hypothesis that mitochondrial stress promotes an adequate balance between tolerance and resistance, protecting the organism from extensive tissue damage and organ failure while still allowing for a proper response towards the invading pathogen to resolve the infection.

2.2. Introduction

Infections are major threats to organismal homeostasis. Adequate immune responses during infection are essential for pathogen clearance and to control inflammation-driven tissue damage. Sepsis results from extreme inflammatory responses to severe infections, followed by immune paralysis and failure of the organism to promote tissue repair strategies. Thus, the hallmark of sepsis is the life-threatening organ dysfunction that is driven by a maladaptive host immune response to infection[1,2].

Current treatment strategies for complex inflammatory conditions focus largely on identifying and controlling the infectious agent and organ support measures[2]. However, sepsis remains a leading cause of death worldwide, and survivors bear life-long consequences since these treatment strategies fail to modulate the host immune response to promote tissue protection and control exacerbated inflammation-driven tissue damage. In fact, a growing body of research has been focused on uncovering strategies for promoting disease tolerance mechanisms[3–6]. Harnessing mechanisms for tissue protection independently from pathogen burden may open new treatment avenues, not only in the field of sepsis but also in many complex multifactorial conditions characterized by extensive tissue damage.

Stress responses constitute highly conserved and critical components of immune surveillance by detecting homeostatic disruptions and initiating appropriate immune responses[7]. Indeed, the deterioration of these responses due to age or disease-associated conditions has been linked with physiological consequences[8]. On the other hand, activating stress responses has been shown to effectively alleviate and even improve organismal responses to challenges and expand lifespan[9].

Mitochondria are essential organelles within a cell to maintain energy rates, metabolic profiles, and cellular signaling. Not surprisingly, several studies have shown a strong association between mitochondrial stress and the induction of protective mechanisms to insults. Importantly, such responses were demonstrated to be crucial in different model organisms, from pioneering work in *C. elegans* revealing that defects in the ETC and inhibition of mitochondrial translation result in lifespan extension[10,11]. Studies in *D. melanogaster* indicate that mitochondrial size, fusion, and fission processes could also promote longevity[12,13]. In agreement, our lab has shown that in a mouse model of bacterial sepsis, mitochondrial stress could promote disease tolerance mechanisms and protect severely septic mice by reducing inflammatory cytokines and organ damage without directly affecting the pathogen[14]. Other independent researchers also confirmed these observations in a model of viral infection[15,16]. Altogether, these data provide compelling evidence that supports the potential of mitohormesis[17].

Stemming from the original concept of hormesis, mitohormesis refers to the adaptive responses triggered upon mild mitochondrial stress that enhance cellular resilience and promote restoration of homeostasis[17,18]. Despite its extensively reported role in lifespan expansion and harnessing tolerance mechanisms, the impact of mitochondrial stress on resistance has been poorly described. In fact, studies showing improvement of innate immune responses associated with mitohormesis have only been described thus far in invertebrate models of *C. elegans*[19,20] and most recently in *D. melanogaster*[21].

In this study, we set out to explore the role of mitochondrial stress in resistance responses in a murine model of sepsis. Following our previous observations that mild mitochondrial stress, induced by phenformin, could harness disease tolerance in bacterial sepsis in mice, resulting in improved survival, lower inflammation, and reduced organ damage scores, we have now gathered evidence showing that after mitochondrial stress, mice can shift the immune response and promote more efficient pathogen clearance. This observation seems to be independent of the gluconeogenic suppressive capacity of phenformin. Furthermore, our results suggest that this is a host-driven response since infecting mice with the pathogen grown in the presence of phenformin did not alter disease severity parameters.

2.3. Methods

2.3.1. Experimental Models

2.3.1.1. Mice

Animal studies were performed following Portuguese regulations and approved by the ethics committee of Instituto Gulbenkian de Ciência, ORBEA, and the national regulatory agency, DGAV, under the project reference number A011.2019. All experiments were performed in the Mouse Facility of the Instituto Gulbenkian de Ciência.

C57BL/6J male mice were obtained from the production unit of the animal facility. All animals used were 8- to 12-weeks-old unless otherwise stated. In all experiments, age-matched animals were randomly assigned to distinct experimental groups.

Animals were housed in specific pathogen-free (SPF) conditions, with 12 hours of light/dark cycles, 50-60 % humidity, and 21-23 ºC temperature. Food and water were available *ad libitum* in all experiments.

2.3.1.2. Bacterial cultures

Unless specified, *Escherichia coli* K12 MG1655 bacteria (from here on, *E. coli*) with resistance to streptomycin was used for *in vivo* infection experiments.

Bacterial cultures were grown in Luria-Bertani (LB) broth supplemented with 100 μg/mL streptomycin (LB + Strep). In experiments assessing the impact of Phenformin on the growth and infection capacity of *E. coli*, bacteria were grown in the presence of streptomycin and 100 μg/mL phenformin hydrochloride (Sigma Aldrich, catalog number P7045).

2.3.2. Methods details

2.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments

For experiments using *E. coli* as an infection trigger, fresh bacterial streaks were made in LB + Strep plates and used within up to one week.

On the day before the infection, a starter culture was prepared from a single *E. coli* colony and grown in LB + Strep liquid media overnight (for 12- to 16 h) at 37 ºC and 180 rpm in a 50 mL reaction tube. On the day of the infection, a second inoculum was prepared in Erlenmeyer's containing a total volume of 10 % of the recipient's capacity, with fresh LB + Strep liquid media and 2 % of the overnight culture.

Bacteria were grown for approximately 2 h 20 min at 37 º C and 180 rpm to reach the early exponential phase ($OD_{600nm} \approx 1$). The culture was centrifuged at 2755 x g and 4 ºC for 6 min, washed with sterile 1 x PBS, centrifuged, and the pellet was then resuspended in the appropriate amount of sterile 1 x PBS for infection.

To induce mild sepsis, the model was optimized for an injection of 200 μL of bacterial suspension with a concentration of $1.75 \times 10^9 – 2.15 \times 10^9$ bacteria/mL (corresponding to an $OD_{600nm} \approx 3.5 – 4.3$). Mice were injected intraperitoneally with the bacterial suspension immediately after preparation with a 27 G needle. Infections were always performed in the morning. Bacterial viability and concentration were confirmed by plating 10^{-6} and 10^{-7} dilutions of the bacterial suspension in LB + Strep plates that were grown overnight at 37 º C.

Phenformin hydrochloride was dissolved in sterile 1 x PBS for intraperitoneal injection of 100 μg/g body weight (administered in 200 μL/mouse), at the time of infection induction.

Mice were monitored for five days (when applicable) for survival, rectal temperature (2, 8, 24, 48, 72, 96 and 120 h), body weight and glucose (every 24 h). Human endpoints were ensured for moribund animals presenting severe sickness phenotypes (i.e. shivering, inability to

maintain an upright position). Tissue collection for various analyses was performed at indicated time points. For this, animals were euthanized by CO_2 inhalation, and organs were collected into appropriate buffers for further experiments or snap-frozen in liquid nitrogen for storage at -80 ºC. Blood was harvested by cardiac puncture with a 25 G needle embedded in 0.5 M EDTA, pH 8.0 to avoid clotting, and for serum collection, centrifuged at 2000 x g and 4º C for 10 min. The serum was snap-frozen in liquid nitrogen and stored at -80 ºC.

Animals that showed no signs of infection (i.e., temperature drop under 35ºC, weight loss of at least 5% at 24 h post-infection, glucose drop under 100 mg/mL) were discarded from experiments.

2.3.2.2. Colony forming units (CFUs) assay

Fresh lung, liver, kidney, and visceral adipose tissue samples were collected into reaction tubes containing 1 mL of sterile 1 x PBS and 2 - 4 tungsten beads for pathogen load assessment of infected mice at indicated time points. Collection tubes were weighed before and after organ collection for normalization calculations. Organ samples were disintegrated in the TissueLyzer II (Qiagen) with two cycles of 4 min at $30s^{-1}$ speed. Circulating blood was collected by cardiac puncture using a 25 G needle without EDTA embedding and 50 µL whole blood was immediately diluted in 450 µL sterile 1 x PBS. In the case of non-terminal pathogen load assessment, tail vein blood was collected by tail nip. Here, 5 µL whole blood were diluted into 45 µL sterile 1 x PBS. For peritoneal lavage fluid collection, mice were disinfected with ethanol after euthanasia by CO_2 inhalation, and an incision was made in the outer skin to expose the inner skin of the peritoneum without rupturing. For CFU assessment, 4 mL of sterile 1 x PBS were injected into the peritoneal cavity with a 27 G needle. The peritoneal area was gently massaged, and the fluid was recovered with a 19 G needle, and the required volume was collected into a sterile 2 mL reaction tube.

Organ and blood pathogen loads were assessed by plating processed samples in LB + Strep plates in serial dilutions ranging from 10^{-1} to 10^{-5}. Colonies were counted after overnight incubation (12- to 16 h) at 37 ºC.

2.3.2.3. Histopathology

For tissue damage assessment quantified by inflammation and necrosis score, fresh lung, liver, and kidney samples from infected mice were collected 24, 72, and 120 h post-infection and treatment. Samples were collected into 10 % (v/v) buffered formalin for fixation. This was followed by embedding in paraffin, sectioning (3 μm), and staining for hematoxylin and eosin according to standard procedures.

An expert pathologist from the histopathology unit at Instituto Gulbenkian de Ciência performed blind tissue damage scoring analysis.

2.3.2.4. Biochemical assays of mouse serum

Serum was collected for cytokine level assessment. The following ELISA kits were used to determine cytokine levels: mouse TNF-α (#430902, BioLegend), mouse IL-6 (#431302, BioLegend), mouse IL-10 (#431411, Biolegend), mouse IL-17A (#432502, BioLegend), mouse IL-12/IL-23 (p40) (#431602, BioLegend) following the manufacturer's instructions. Absorbance readings were performed on 96-well plates using the Infinite M200 plate reader (Tecan). Data was processed on the online free tool GainData® arigo's ELISA calculator from Arigo Laboratories.

2.3.2.5. E. coli growth curves *in vitro*

To test the impact of phenformin on *E. coli* growth, *in vitro* growth curves were performed. For this, a single colony of *E. coli* was picked from a freshly streaked plate and grown overnight in liquid LB + Strep at 37 º C, 180 rpm. On the following day, 2 % (v/v) of the overnight *E. coli* culture was plated in a 96-well plate in fresh LB media containing different concentrations of phenformin hydrochloride, ranging from 100 μg/mL to 500 μg/mL, and control conditions. Three independent experiments were performed, each with three biological and technical replicates. Bacteria were grown in these conditions for 24 h at 37 º C, shaking using the Synergy H1 microplate reader (BioTek). Absorbance measurements at OD_{600nm} were performed every 2 min.

2.3.2.6. Data modeling and statistical analysis

All data was analyzed using R studio software. For visualization, the ggplot2 and ggpubr packages were used.

For survival analysis, Cox proportional hazard linear regression models, with random effect structure (1|Experiment), was adopted using the Coxme package. When the experimental layout required sacrificing groups of animals at specific time points, data was analyzed with censored events (including only groups of animals not sacrificed because of experimental read-outs). This criterion was also employed for visualization in representative plots.

For vital parameters analysis, including temperature, weight, and glucose, linear regression, with random effect structure (1|Experiment), one per time-point was performed. This prevents the model from estimating parameters for animals that have succumbed to disease and thus cease to exist at later time points.

For pathogen loads, CFUs were analyzed by modeling the effect of treatment across the different time points for each independent experiment through mixed-effect zero-inflated negative binomial regression.

Histopathology scores, as the data is based on a Likert scale (0 - 5), were analyzed by ordinary logistic regression and Fisher and Chi-square tests. Cytokine levels were analyzed with T-test for normally distributed groups and Wilcoxon's test as a non-parametric alternative. Growth curves were analyzed by linear regression.

2.4. Results

2.4.1. Bacterial peritonitis model optimization for mild sepsis using E. *coli*

To explore the potential of mild mitochondrial stress in homeostasis recovery after an insult, we began by optimizing a bacterial peritonitis model to phenocopy mild sepsis. For this, we aimed at a 50 – 75 % survival of infected mice up to 5 days post-infection, with animals presenting clear signs of infection, namely temperature drop under 35 ºC and/or weight loss of at least 5 % at 24 h post-infection. To achieve this, we injected animals with different concentrations of *E. coli*, using the previously published lethal model, in the work from Colaço and colleagues as a baseline[14] and by administering lower concentrations to reduce the severity of infection to the intended degree. Infected mice were followed for 5 days (120 h).

Vital parameters were collected up to 72 h post-infection, as well as tail vein blood, to assess circulating blood pathogen load. At 5 days post-infection, surviving animals were sacrificed and their organs were collected for pathogen load assessment by colony forming unit assay as described in methods described in 2.3.2.2.. Results are shown in Figure 2.1.

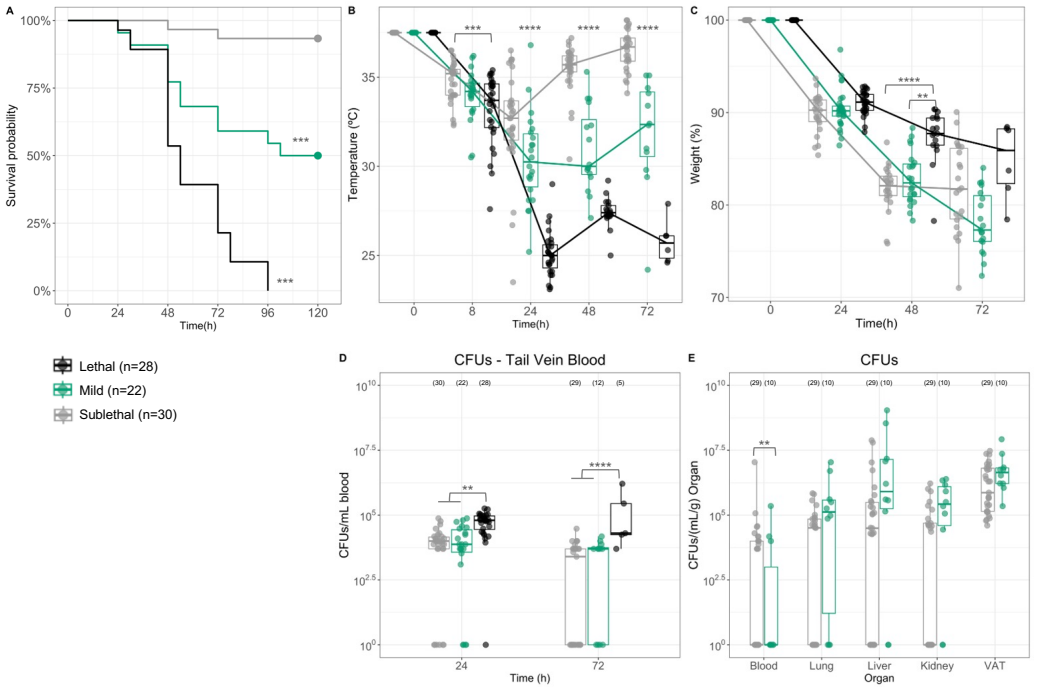

Figure 2. 1- Bacterial peritonitis model optimization.

Survival **(A)**, rectal temperature **(B),** and % initial body weight **(C)** after infection of male C57BL/6J mice infected with different streptomycin-resistant *E.coli* concentrations at 0, 24, 48, and 72 h. Groups are divided in **lethal (colored in black**), for animals injected with more than 4.5 x10^8 CFU/mouse, **mild (colored in green)**, for animals injected with 2.5 - 4.5 x10^8 CFU/mouse, and **sublethal (colored in grey**), for animals injected with less than 2.5 x10^8 CFU/mouse (**D)** Bacterial load from mouse tail vein blood at the indicated time-points after infection. (**E)** Bacterial load from mouse blood and organs after terminal collection at 120 h post-infection, from the left to the right, cardiac blood, lung, liver, kidney, and visceral adipose tissue (VAT) bacterial loads. **(A-E)** represent pooled data from 2 independent experiments; circles represent individual mice, box plots indicate quartiles and median. **Statistical analysis** was done using R by modeling the effect of treatment across the different time points independent of the experiment. Survival was analyzed by Cox proportional hazard linear regression corrected for experiment, where the animals alive and sacrificed at the time points 24 h and 72 h were disregarded. Temperature, and weight were analyzed linear regression corrected for experiment. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Results show that to obtain a 50 – 75 % survival after 120 h of infection, animals should be injected with 2.5 – 4.5 x 10^8 CFU/mouse (mild dose) (Figure 2.1 (A)). We also observed that with injections higher than 4.5 x 10^8 CFU/mouse (lethal dose), no animals survive until 120 h post-infection, and at 48 h, approximately 50 % will have succumbed to the infection. In contrast, if the injection is less than 2.5 x 10^8 CFU/mouse (sublethal dose), survival is around

90 % at 120 h post-infection. Regarding vital parameters, we can observe a clear correlation between the severity (in terms of survival) and the temperature drop in each group. Animals injected with the lethal dose show much greater temperature drops than mild and sublethal dose mice (Figure 2.1 (B)). Lastly, if we consider weight drop sublethal and mild mice seem to show similar kinetics of reduction of weight until 48 h, however at 72 h, mild dose animals have a lower weight than sublethal mice, which provides evidence for a higher degree of disease severity in the mild mice when compared to sublethal (Figure 2.1 (C)). Lethal group mice do not show such great reductions in weight. This might be due to the reduced number of animals per group at this later time point, which in turn, biases this measure for animals that might respond better to the infection. Another reason for lower weight loss in the lethal group could be linked to the greater temperature drop these mice have compared to the other groups.

To fully ascertain that we have phenocopied a mild sepsis model, we have also looked at tail vein blood pathogen load throughout the experiment and at organ pathogen load through the terminal collection at 120 h (Figure 2.1 (D and E)). These results support the association between the severity of disease observed by vital parameter assessment and the groups' pathogen burden. Albeit there are no animals from the lethal group at 120 h to compare the pathogen burden of lethal with mild and sublethal doses in the organs, we can see through the tail vein blood that these mice had higher pathogen burden throughout the experiment. Organ pathogen assessment further supported that mild-dose mice have higher disease scores which can be correlated with higher pathogen burden.

2.4.2. Phenformin-induced mild mitochondrial stress confers protection against bacterial sepsis

To explore the role of host-driven protective mechanisms triggered by mild mitochondrial stress, we treated mild septic mice with 100 μg/g body weight phenformin by intraperitoneal injection at the time that infection was induced. For this, we used the model of mild infection optimized and described in this chapter, results from section 2.4.1.. Mice were followed for 120 h, and disease severity was assessed in both groups.

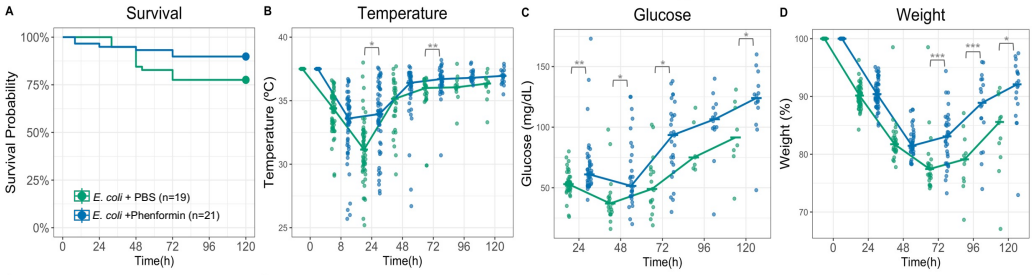

Figure 2. 2 - Mild mitochondrial stress protects mice from mild sepsis.

Survival **(A)**, rectal temperature **(B),** glucose **(C)**, and % initial body weight **(D)** after infection of male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at 0, 24, 48, 72, 96 and 120 h. (A-D) represents pooled data from 4 independent experiments. (A) Survival plots exclude data from groups of animals sacrificed at 2, 24, and 72 h for other experimental purposes. (B-D) represent individual animals pooled from 4 independent experiments (n = 58 for E. coli+PBS, n=59 for E. coli+Phen). **Statistical analysis** was done using R by modeling the effect of treatment across the different time points independent of the experiment. Survival was analyzed by Cox proportional hazard linear regression corrected for experiment, where the animals alive and sacrificed at the time points 24 h and 72 h were disregarded. Temperature, glucose, and weight were analyzed by linear regression corrected for the experiment. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Results (Figure 2.2) show that phenformin treatment improves survival and contributes to a significantly faster disease recovery rate. This is notably observable in all vital parameters, with temperature and glucose level recoveries already at 24 h post-infection and treatment (Figure 2.2 (B-C)), and greater body weight recovery of treated mice from 72 h onwards (Figure 2.2 (D)). These results support that phenformin can confer protection to bacterial sepsis in a milder model, in addition to the already protective mechanisms described for acute bacterial sepsis by our lab, in Colaço and colleagues' work[14].

2.4.3. Disease resistance and tolerance mechanisms confer protection to phenformin-treated septic mice

To elucidate the mechanisms underlying the faster disease recovery observed in septic mice treated with phenformin, we then proceeded to assess pathogen burden levels across the body (Figure 2.3), tissue damage scores, and circulating inflammatory markers (Figures 2.4 and 2.5) of infected treated and control mice as key indicators of disease resistance and tolerance mechanisms respectively.

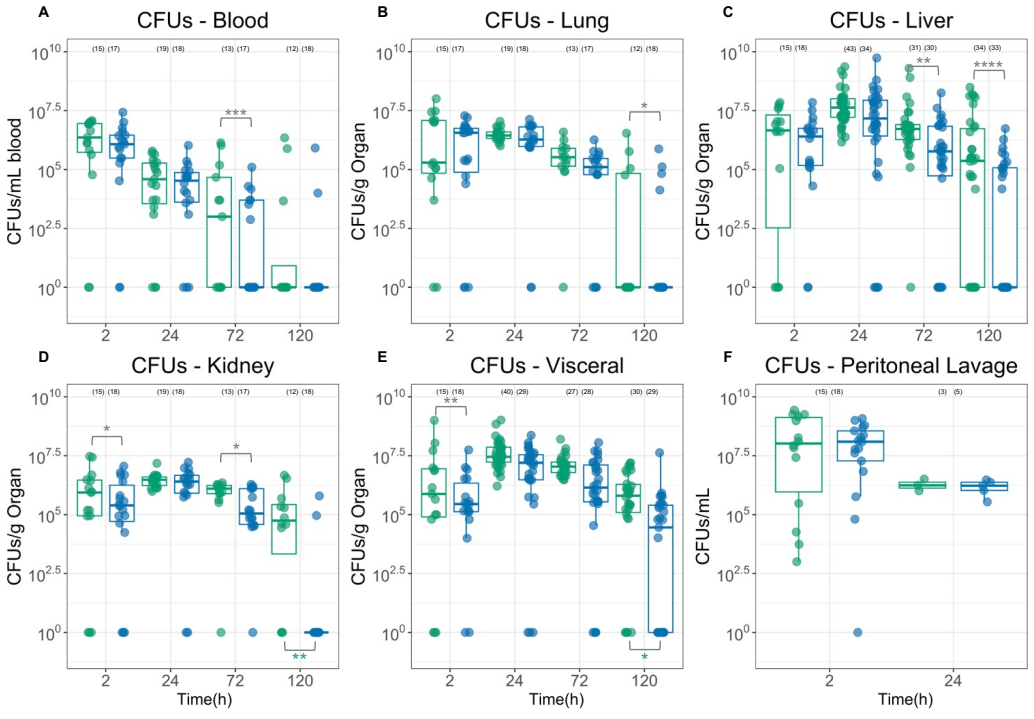

Figure 2. 3 - Phenformin-induced resistance promotes pathogen clearance mechanisms.

Bacterial load in mouse **(A)** blood, **(B)** lung, **(C)** liver, **(D)** kidney**, (E)** visceral adipose tissue**, (F)** peritoneal lavage at the indicated time-points after infection. **(A-F)** circles represent individual mice from at least three independent experiments, box plots indicate minimum, maximum, and mean. **(A-F)** represent pooled data from at least 3 independent experiments; squares represent individual mice and box plots indicate quartiles and median. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time points for each independent experiment. CFUs were analyzed by mixed-effect zero-inflated negative binomial regression. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****) in which black * indicate differences in CFU counts and teal blue * indicate differences in zero CFUs.

Our data indicates that 2 h after infection, peritoneum, blood, lung, and liver bacterial burdens are similar between groups. Phenformin-treated mice show slightly reduced pathogen levels in the kidney and visceral adipose tissue. However, at 24 h post-infection, bacterial burdens have stabilized, and results show comparable bacterial levels in treated and non-treated mice in all organs. At 72 h post-infection, phenformin-treated mice have significantly reduced pathogen loads in the blood, liver and kidney. At 120 h, we observe significant differences between treated and non-treated animals in their bacteria-clearing capacity as assessed by the number of animals that show non-detectable levels of bacteria in their organs. This is observable in all tissues but markedly visible in the visceral adipose tissue and kidney, in which infected non-treated mice still display high levels of bacterial loads, while a significant number of phenformin animals show no bacterial count or very reduced pathogen loads.

Interestingly, despite presenting similar bacterial burdens at 24 h, phenformin-treated mice have reduced tissue damage markers, as demonstrated by blind histopathology analyses of the lung and liver (Figure 2.4) and cytokine levels (Figure 2.5). We found generally less inflammation in the lungs visible at 24 and 72 h, and in the liver of treated mice, more significantly at 72 h post-infection and treatment. Additionally, cytokine profiling indicated reduced levels of pro-inflammatory cytokines, such as IL-6, IL-12/13, IL-17A, and TNFα in treated mice 6 h post-infection.

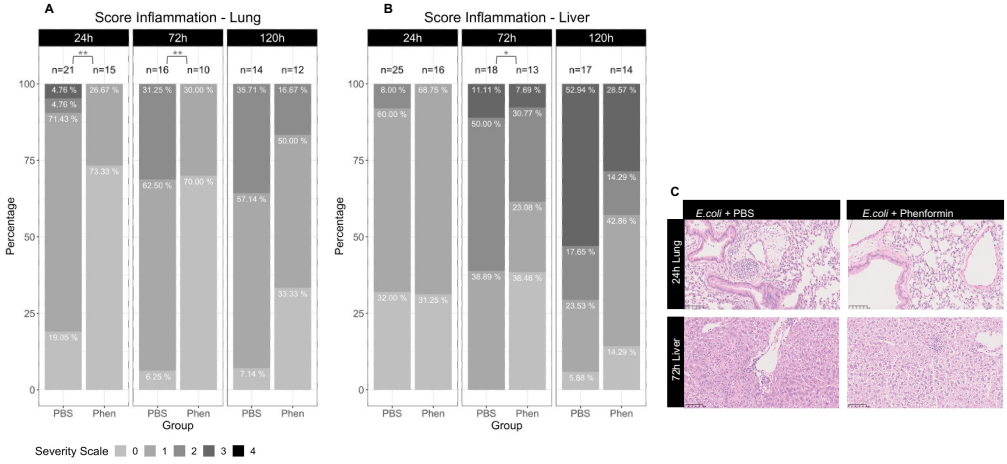

Figure 2. 4 - Phenformin-induced tissue protection results in lower tissue damage scores.

(A-D) Organ damage score in Hematoxylin-eosin-stained tissues 24, 72, and 120 h after infection of male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). Score 0 = no lesions; 1 = very mild; 2 = mild; 3 = moderate; 4 = severe lesions. Data represents at least three independent experiments. **(C)** Representative images Hematoxylin-eosin-stained lung 24h after infection and liver 72h after infection and administration of treatment or vehicle. Scale bars indicate 200 mm (lung) and 500 mm (liver). **Statistical analysis** was done using R, using the Fisher Exact Test. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).

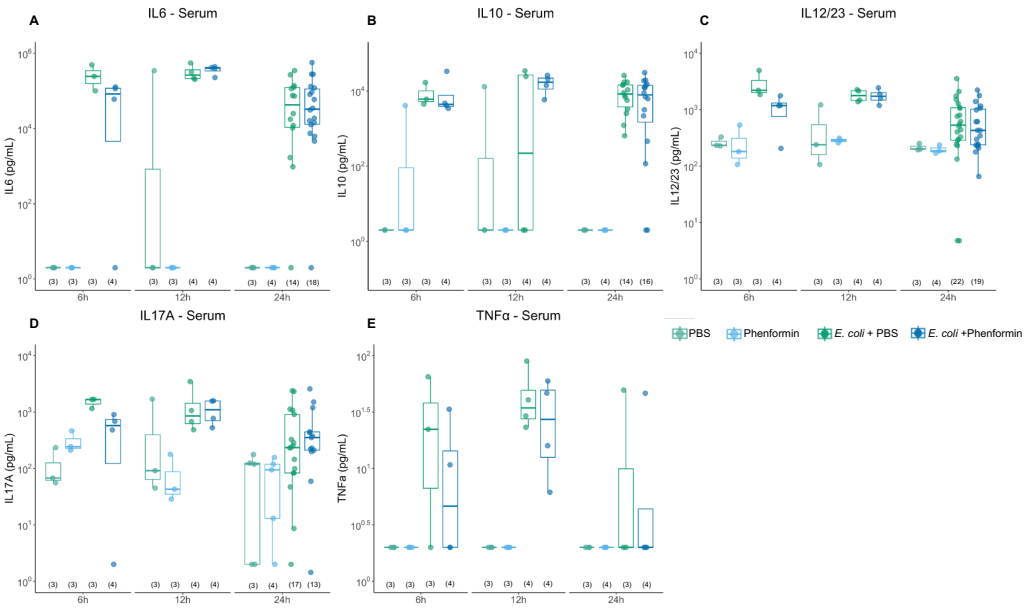

Figure 2. 5- Cytokine profiles in circulating blood during infection and treatment.

(A-E) Concentrations of Interleukins -6, -10, -12, -17A and TNFα (IL6, IL10, IL12, IL17A and TNFα) in serum samples of male C57BL/6J mice 6, 12, and 24 h non-infected or post-infection with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A-E)** represent one experiment for the time points of 6 and 12 h and pooled data from at least 3 independent experiments for the 24 h time point. Individual mice measured are represented by points, and box plots indicate quartiles and median. **Statistical analysis** was done using R, using the t-test for normally distributed groups, and Wilcoxon's test as a non-parametric alternative. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Altogether, these results suggest that phenformin treatment of septic mice confers protection at first by promoting the prevalence of disease tolerance mechanisms. This enables the control of the immune response to prevent the exacerbation of the inflammatory response, a hallmark of a detrimental phenotype in sepsis. Later, after 48 h, resistance mechanisms become more predominant, and the host response adapts towards pathogen clearance, promoting restoration of organismal homeostasis.

2.4.4. Phenformin does not impact *E. coli* infection capacity

This work uses a mitochondrial targeting drug to study potential host-driven mechanisms to recover homeostasis after an insult. Considering we use *E. coli* to trigger sepsis, and the close evolutionary connection between mitochondria and bacteria[22], we investigated whether phenformin had any direct effect on the bacteria we were using to induce the infectious and

inflammatory condition. For this, *in vitro* growth curve experiments were performed as described in methods 2.3.2.5.. Here, streptomycin-resistant *E. coli* was grown for 24 h in LB media and in the presence of phenformin in different concentrations. Phenformin concentrations ranged from 100 μg/mL (direct equivalent to what we use in *in vivo* studies) and 500 μg/mL. Bacteria density was measured at OD_{600nm} every 2 min. Results are shown in Supplementary Figure 2.1.

Our data indicated that phenformin slightly impacts *E. coli* growth rates *in vitro* and the magnitude of the impact is proportional to the concentration of the drug. However, extensive literature shows that *in vitro* bacterial growth can misrepresent what occurs *in vivo*[23]. This could be due to differences in nutrient availability and surrounding environment conditions, which can ultimately play a role in biofilm formation, or due to host responses[23]. Not surprisingly LB broth and the peritoneal cavity have very different nutrient compositions. In fact, a recent study has shown that biguanides, such as metformin and phenformin, seem to improve *E. coli* capacity to hijack the uptake B12 in the peritoneal cavity[24]. Vitamin B12 uptake by *E. coli* has been associated with a reduction of lag phase and accelerated growth rate[25]. Therefore, indicating that the results obtained *in vitro* might not be representative of a possible impact of phenformin on the *E. coli in vivo.*

The strain of *E. coli* used in this work (*Escherichia coli* K12 MG1655) has been extensively studied and adapted in the laboratory and does not secret virulence in mice[26,27]. Thus, our infection model mostly relies on a host response towards elevated pathogen burden in the body, independently of virulence factors. Considering this, the reported role of phenformin in B12 uptake by *E. coli,* and its association with faster growth rates, we decided to assess whether growing *E. coli* in the presence of phenformin would impair the capacity and extent of inducing disease symptoms and sepsis.

To address this question, we grew *E. coli* in control conditions or, in the presence of 100 μg/mL of phenformin. This was done for both the overnight inoculum and the second inoculum for the infection (as described in methods 2.3.2.1).

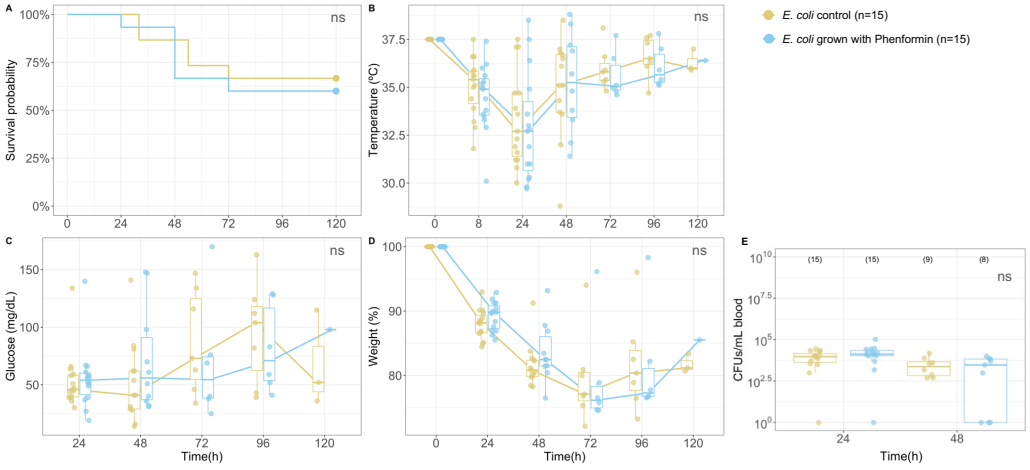

Figure 2. 6 - The infection capacity of E. coli is not impacted by phenformin.

Survival (A), rectal temperature (B), glucose (C), and % initial body weight (D) after infection of male C57BL/6J mice with 4 x 108 CFU/mouse streptomycin-resistant *E. coli* grown in the presence 100 µg/mL phenformin (colored light blue) (or only streptomycin as a control - colored in light yellow) at 0, 24, 48, 72, 96 and 120 h. (E) Bacterial load from mouse tail vein blood at the indicated time points after infection. (A-E) represent pooled data from 3 independent experiments; circles represent individual mice, box plots indicate quartiles and median. Statistical analysis was done using R, by modeling the effect of treatment across the different time-points independent of the experiment. For survival analysis, the animals alive and sacrificed at the time-points 24h and 72h were disregarded. Temperature, glucose, and weight were analyzed by linear regression corrected by experiment. CFUs were analyzed by mixed-effect zero-inflated negative binomial regression. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****), not-significant (ns).

Our results show that the presence of phenformin during the growth of *E. coli* does not impact the capacity of the bacteria to infect mice and trigger sepsis. Both bacteria inoculates yield similar mortality rates. In fact, mice infected both with control, or phenformin-grown *E. coli* show similar disease severity parameters as demonstrated by similar temperature (Figure 2.6 (B) and glucose drop curves (Figure 2.6 (C)) and body weight loss (Figure 2.6 (D)). Furthermore, tail vein blood pathogen loads indicate that both groups have comparable pathogen burden levels at 24 h post-infection (Figure 2.6 (E)).

Together, these experiments validate that phenformin does not impact the capacity of *E. coli* to infect mice. This is important evidence for our work, to disentangle the consequences of our treatment on the host – thus allowing us to elucidate host-driven responses from possible side-effects on the pathogen used to cause the inflammatory condition.

2.4.5. Phenformin gluconeogenesis suppressive capacity is normalized within 8 h of treatment

One of the most studied characteristics of biguanides, such as phenformin, is their capacity to suppress gluconeogenesis and lead to blood glucose reduction[28]. Considering that we have observed lower glucose levels associated with the disease severity of the infection and an improved phenotype with phenformin treatment of infected mice, it becomes important to disentangle the effect of gluconeogenesis suppression of phenformin treatment from the blood glucose lowering associated with our sepsis model. For this purpose, we injected male C57BL/6J mice with 100 μg/g body weight phenformin or vehicle control solution. We then measured blood glucose levels of these mice by tail vein blood collection, every 2 h for a period of 8 h, and at 24- and 48 h post-injections.

Results (Figure 2.7) show that phenformins' capacity to suppress gluconeogenesis peaks at 2 h post-injection. This is in line with what was first reported in 1960, showing peak phenformin concentration in the liver at 2 h post intraperitoneal injection[28,29]. Additionally, we have observed that 8 h after phenformin treatment, blood glucose levels of treated mice have recovered to homeostatic concentrations.

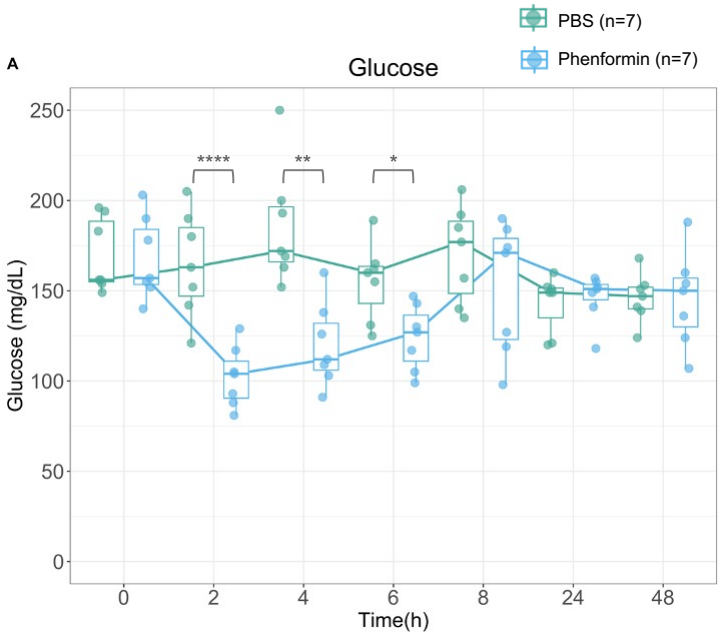

Figure 2. 7 - Gluconeogenesis suppressive capacity of phenformin.

Glucose measured from tail vein blood after injection of male C57BL/6J mice with 100 μg/mL phenformin (**light blue**) or control vehicle solution (**light green**) (PBS). Data represents one individual experiment, circles represent individual mice, box plots indicate quartiles and median. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time-points. Glucose was analyzed by linear regression. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

2.5. Discussion

Growing evidence supports the potential of inducing stress responses to restore homeostasis in complex multifactorial inflammatory conditions, such as sepsis[14,16,30,31].

As a predominately host-driven condition, modulation of host responses during sepsis by stress-induced mechanisms must be understood in depth to avoid skewing the host responses detrimentally. Previous work in our laboratory has shown that severely septic mice are protected from acute bacterial sepsis through the induction of stress responses via the administration of mitochondrial targeting drugs[14,32]. This protection was described mainly to be conferred by the induction of disease tolerance mechanisms, through which mice present significantly lower tissue damage despite similar pathogen loads. However, our previous study's bacterial peritonitis sepsis model was limited to a severe infection model, where over 50 % of mice were already dead at 48 h post-infection. In an infection model with this severity, it is impossible to monitor disease progression sufficiently long to properly dissect all the active processes that deal with the infection. Considering that resistance mechanisms could require more time, it is possible that we have not observed any impact on resistance thus far due to the fast lethality of the previous model.

To truly understand the potential of using mitochondrial targeting drugs to promote restoration of homeostasis, it is necessary to dissect the impact of these treatment strategies in the balance between disease tolerance and resistance mechanisms. Namely, we aimed to elucidate whether tolerance mechanisms are being promoted to the detriment of the resistance responses or whether mitochondrial targeting could be a platform to modulate host responses for a proper balance between inducing tissue protection and pathogen clearance.

To address this question, we optimized our murine sepsis model to monitor septic mice for longer time frames. This has allowed us to study in more detail the impact of mitochondrial targeting by phenformin administration on host responses.

Results from this work report that mild mitochondrial stress protects septic mice by favoring disease tolerance mechanisms at first, as described in Colaço *et al.*[14]. Here, we observe reduced tissue damage scores up to 48 h without any significant change in pathogen loads. However, using the mild sepsis model we can also observe that 72 h after infection and treatment, host responses shift from tolerance to resistance mechanisms, and results report an improved bacterial clearance in treated animals.

To induce mild mitochondrial stress, we rely on phenformins' ability to inhibit complex I of the mitochondrial electron transport chain, as described in our previous work[14]. However, phenformin has other organismal effects that have been more extensively studied and characterized. In fact, phenformin was first used in the clinics as an anti-diabetic treatment due to its capacity to suppress gluconeogenesis[28]. Not only is the process of gluconeogenesis tightly connected to the prognosis in sepsis patients, but it is also highly associated with pathogens' infectious capacity, virulence, and burden to the host[33–35]. Thus, we aimed to explore the extent of gluconeogenesis suppressive capacity of phenformin to disentangle this effect of our treatment from its capacity to modulate disease tolerance and resistance mechanisms.

Results show that our treatment strategy triggers gluconeogenesis suppression in non-infected mice, peaking at 2 h post-injection. After 8 h, phenformin-treated animals do not show gluconeogenesis suppression, presenting similar blood glucose levels as their control counterparts. Considering our results from infected mice, we can speculate that the disease tolerance and resistance phenotypes we have observed are independent of phenformins' capacity to suppress gluconeogenesis.

Lastly, considering the close evolutionary ties between eukaryotic mitochondria and bacteria, as highlighted by the endosymbiotic theory[22,36], it becomes important to elucidate whether phenformin can have any direct impact on our sepsis-driving pathogen, the *E. coli* strain used to induce infection. Thus, in this chapter, we have evaluated by *in vitro* and *in vivo* methods, whether phenformin could impact (1) the rate of *E. coli* growth and (2) the ability of *E. coli* to infect mice and trigger sepsis. Our results have shown that the dose used in the present study does not impact the growth or infection ability of our bacteria *in vivo*. Thus, suggesting that the phenotypes observed in this study are a direct consequence of phenformins' capacity to modulate the host-response independently of the pathogen.

Together, the results of this chapter indicate that mild mitochondrial stress, triggered by a single injection of phenformin, can modulate the host response during sepsis, favoring a beneficial balance between disease tolerance and resistance. This results in the control of the exacerbated immune response, characteristic of sepsis, here observable through the lower tissue damage scores, and a more efficient clearing capacity of the pathogen, observed by the faster reduction and ultimately the complete clearing of pathogen loads from organs of treated mice. To further elucidate the protective mechanisms triggered by mild mitochondrial stress, we have proceeded by immunophenotyping cell populations in organs of interest in this sepsis model. In Chapter 3, we will describe the changes in immune cell populations we have observed.

Acknowledgments

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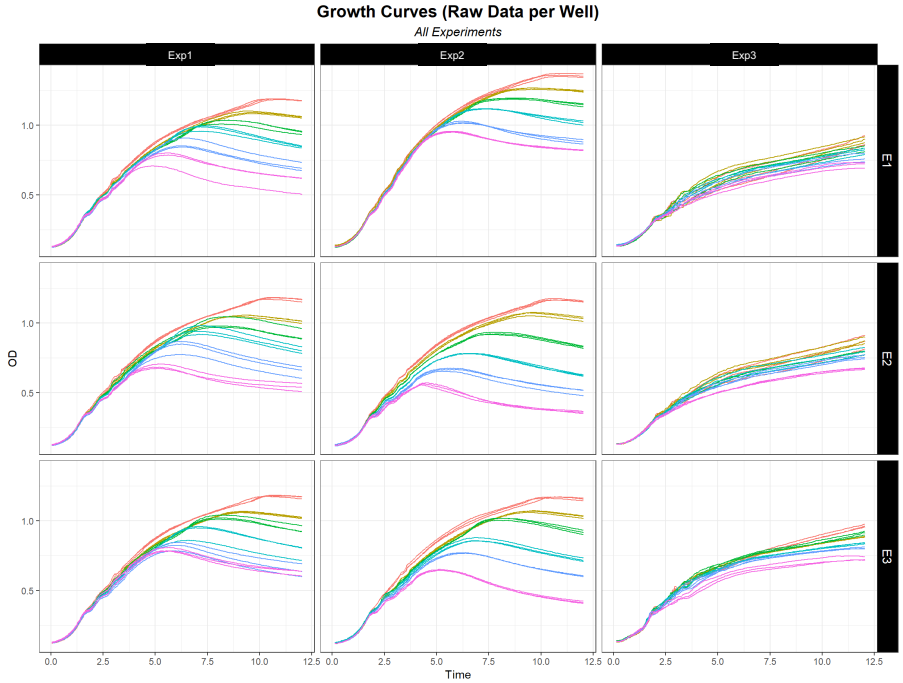
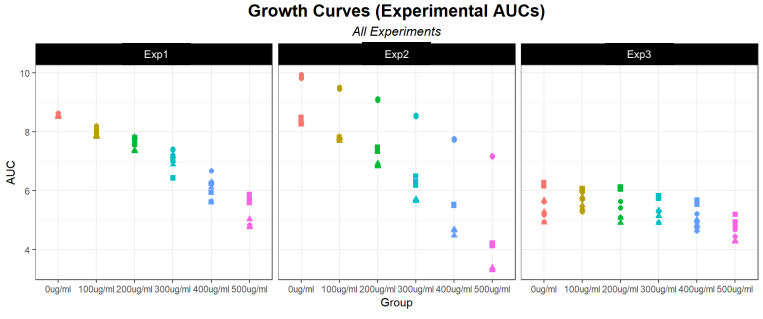
2.7. Supplementary data





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formula	contrast	estimate	adj.p.value	
~0+replicate+Group	(100ug/ml) - (0ug/ml)	-0.3126898	0.1360234	
	(200ug/ml) - (0ug/ml)	-0.6904139	0.0000286	****
	(300ug/ml) - (0ug/ml)	-1.2384889	0.0000000	
	(400ug/ml) - (0ug/ml)	-1.9329630	0.0000000	
	(500ug/ml) - (0ug/ml)	-2.7105722	0.0000000	

Supplementary Figure 2. 1 - Growth curves of the Streptomycin resistant E. coli strain in the presence or absence of 100, 200, 300, 400, 500 µg/mL phenformin.

(A) Optical density measures (ODs) raw data of three independent experiments each with three biological replicates each. **(B) Area under the curve (AUCs)** results represent three independent experiments with three biological replicates each. **(C)** Summary of linear regression of AUCs values, considering replicate and group as

variable factors. **Statistical analysis** was done using R, by linear regression. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Chapter 3

Phenformin-induced modulation of the immune response is organ- and cell-type-specific

Author contributions

Kátia Jesus was responsible for planning, executing all experiments, and running analysis scripts on data from all experiments in this chapter. André Barros, Elsa Seixas, and Fikir Asfaw provided assistance in the *in vivo* work. André Barros developed the scripts for statistical analysis. Miguel Mesquita assisted in the design of flow cytometry gating strategies. Luís Ferreira Moita supervised the project and reviewed this chapter.

3.1. Abstract

One of the major concerns in sepsis is the hosts' exacerbated and dysregulated immune response that ultimately causes extensive and life-threatening organ failure resulting from tissue damage. With this in mind, the field has been invested in finding novel therapeutic interventions to protect and improve the outcomes of septic patients. Our lab has gathered compelling evidence showing that the induction of stress pathways may be a potential therapeutic approach for modulating host responses. This work raised the possibility that mild mitochondrial stress could be used to control the exacerbation of the immune response by decreasing tissue damage and the risk for organ failure while also showing the potential to be a platform to allow for a proper immune response to neutralize and clear out the pathogen.

Here, we used the mouse model of bacterial sepsis described in the previous chapter and, using flow cytometry, characterized the immune system changes modulated by mitochondrial stress induced by the administration of phenformin throughout the organism and during infection.

We found that phenformin modulates immune cell dynamics in an organ- and cell-type-specific manner. The protective phenotype of phenformin seems to depend on boosting the recruitment of neutrophils to the site of infection, promoting peritoneal monocyte recruitment and consequent macrophage replenishment while at the same time preventing an exacerbated influx of neutrophils to the lung. On the other hand, phenformin lowers lymphocyte activation marker CD69 expression, possibly contributing to a decrease in the egress of lymphocyte populations from the spleen following infection.

3.2. Introduction

The immune system is an extremely complex and tightly regulated system that has evolved to protect organisms from internal and external insults. When an insult is sensed, an immune response is built that aims to neutralize the threat and repair the damage.

Specifically, when an infection occurs, host responses that sense microbe-associated molecular patterns (MAMPs) and/or danger-associated molecular patterns (DAMPs), recruit innate immune cells, such as neutrophils and macrophages, to infection sites to control pathogen growth and migration to other organs. Innate immune cell functional activation, as well as pro-inflammatory adaptive immune cell recruitment, are mediated by pro-inflammatory cytokines, which include TNF, IL-1β, and IL-6. Importantly, upon pathogen clearance, a

functional reprogramming of cells needs to occur for homeostasis recovery. Herein, mediators of inflammation need to be replaced by anti-inflammatory signals. The production of IL-10 by regulatory T cells and IL-37 and TGF-β, released by monocytes and platelets, respectively, mediate this shift in immune programs[1].

Severe infections and complex inflammatory conditions impose profound changes in host metabolism and energy demand, ultimately reflected in immune cell responses. As a result, immune system dynamics can be altered during an insult, and in extreme conditions, these alterations could be highly detrimental and difficult to reverse.

Sepsis is a prime example of an extreme condition resulting from extensive changes triggered by an insult[2]. This condition, usually triggered by a pathogen infection, consists of a maladaptive immune response of the host. It is characterized by a pro-inflammatory burst that exacerbates immune system dynamics, followed by an immune paralysis linked to the exhaustion and senescence of the immune system[2]. This hinders the required tissue regeneration programs and ultimately results in lifelong-lasting tissue and organ damage.

Compelling evidence from our lab and other researchers suggests that inducing stress responses by mild disruptions to core cellular functions could be a potential tool to promote disease tolerance mechanisms during infection[3–7]. More specifically, mild mitochondrial stress has been shown to improve lifespan in invertebrate and vertebrate models and control exacerbated immune responses to an insult by highly evolutionary conserved mechanisms[8–10].

The role of mitochondria in cellular and organismal homeostasis is highly diverse. From its widely studied and most known role in energy metabolism to regulation in signaling cascades, development, and differentiation[11,12]. Mitochondria homeostasis and, concomitantly, its disruption can have significant impacts on physiological processes and disease pathogenesis. Mitochondria have been described to have a role in metabolic disorders. For example, in insulin resistance associated with type 2 diabetes, cardiovascular disease[13], impaired mitochondria function has been shown to lead to oxidative stress, calcium dysregulation and neuronal death in neurodegenerative diseases[14,15]. Regulation of cell growth by the mitochondria has also been linked with cancer progression[16] and accumulation of mitochondrial DNA contributes to promoting age-related diseases[17]. In line with this, mitochondrial dysregulation in the immune system can have distinct impacts depending on the cell population.

Immune cells have different energetic requirements. Depending on their function and activation state, immune cells can rely more on aerobic glycolysis, tricarboxylic acid (TCA) cycle, or oxidative phosphorylation. Furthermore, depending on external conditions and stimuli, the main bioenergetic program can also change[18]. For example, macrophages with a pro-inflammatory profile exhibit a broken TCA cycle and upregulate glycolysis, while alternatively activated macrophages utilize β-oxidation of fatty acids and oxidative phosphorylation (OXPHOS) as their main energy source[19–21]. In line with this, resting dendritic cells mostly use fatty-acid oxidation (FAO), whereas activation of these cells triggers a metabolic switch to resort to glycolysis[22].

On the other hand, neutrophils rely fully on glycolysis due to their rapid and energetically demanding effector functions[23]. Lymphocytes can be divided into several different subsets with varying energetic needs. Non-activated T cells are metabolically quiescent, their glycolysis levels are very low, and energy requirements are mainly maintained by FAO and/or OXPHOS. On the other hand, activating these subsets triggers a metabolic switch towards using glycolysis and the TCA cycle to meet the energetic needs[24–26]. Natural killer (NK) cells are at the intersection between innate and adaptive immune cells and have a constant high energy demand to fulfill their effector functions. At rest, NK cells preferentially make use of OXPHOS, however upon activation, glycolysis and OXPHOS are both greatly increased[27,28].

These different energetic needs ultimately reflect different, if not even opposing, phenotypes elicited in innate and adaptive immune cells upon mitochondrial dysfunction. Studies have shown that mitochondrial metabolism is involved in fate decisions in T cell differentiation[29,30]. Moreover, a study using phenformin, which inhibits complex I of the mitochondrial electron transport chain (ETC), has shown that the modulation of T cell populations with treatment can delay the development of T cell acute lymphoblastic leukemia (T-ALL)[31]. On the other hand, mitochondrial oxidative stress is involved in neutrophil extracellular trap (NET)[32] release [33], and complex I deficiency in microglia was shown to induce neuronal dysfunction and early lethality[34–36].

We have previously shown that complex I ETC inhibition by phenformin could protect severely septic mice through disease tolerance mechanisms, preventing tissue damage and organ failure[3]. Additionally, this work has highlighted evidence that these same stress responses can modulate resistance and promote a more efficient pathogen clearance and infection resolution. This led us to hypothesize that mild mitochondrial stress could influence immune dynamics during sepsis.

Here, we used flow cytometry to characterize the immune cell population dynamics throughout the time course of infection in a systemic way. We found that phenformin seems to control the exacerbation of lymphocyte and NK cell activation by rapidly controlling levels of activation marker CD69 after infection. On the other hand, treatment induces preferential recruitment of neutrophils to the peritoneal cavity during infection, preventing exacerbated recruitment of these cells to the lung. Tissue-resident macrophage populations of the peritoneal cavity undergo Macrophage Disturbance of Homeostasis Reaction (MDHR) upon phenformin administration, which conditions the recruitment of monocytes. This ultimately provides a stable influx of monocytes to the peritoneal cavity early on during infection, translating to a faster recovery of macrophage populations essential for *E. coli* clearance.

3.3. Methods

3.3.1. Experimental Models

3.3.1.1. Mice

See section 2.3.1.1..

3.3.1.2. Bacterial cultures

See section 2.3.1.2. .

3.3.2. Methods details

3.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments

See section 2.3.2.1. .

3.3.2.2. Flow cytometry sample preparation

Immunophenotyping of cell populations was performed in blood, peritoneal cavity, lung, and spleen. Samples were collected at the indicated time points (8, 24, and 72 h post-infection and treatment) and processed as described.

Circulating blood was collected by cardiac puncture after euthanasia by CO_2 inhalation with a 25 G needle previously embedded in EDTA to avoid clotting. Whole blood samples were pipetted into a 96-well plate. Blood cells were pelleted by centrifugation at 320 x g and 4 ºC for 5 min, the supernatant was discarded, and the pellet was resuspended in red blood cell lysis (RBC) buffer (155 mM ammonium chloride, 12 mM sodium bicarbonate, 0.1 mM EDTA). Cells were briefly incubated (2 – 5 min), and FACS buffer (1 x PBS, 2 % FBS, 0.02% Sodium azide) was added to wash; cells were centrifuged and washed one more time before staining.

For peritoneal cavity cell collection, mice were disinfected with 70 % ethanol after euthanasia by CO_2 inhalation, and an incision was made in the outer skin to expose the inner skin of the peritoneum without rupturing. For cell recovery, 4 mL of sterile 1 x PBS (when samples were used in parallel for CFU or biochemical assays) or FACS buffer was injected into the peritoneal cavity with a 27 G needle. Peritoneal cells were dislodged by massaging the area. Peritoneal fluid was recovered with a 19 G needle. When required, a red blood cell lysis step followed peritoneal cavity cell recovery. Otherwise, samples were centrifuged and resuspended in FACS buffer for staining.

Lung samples were collected after perfusion with 10 mL sterile 1 x PBS using a 25 G needle and a 10 mL syringe. The tissue was minced, placed in a 50 mL reaction tube in digestion buffer (HBSS, 2 mg/mL DNAse I, 3 mg/mL collagenase I), and incubated for 45 min at 37º C shaking. After incubation, samples were placed on ice, and FACS buffer was added to dilute the enzymes. Samples were filtered through a 40 μm nylon filter, and cells were ground using a plunger. Cell suspensions were centrifuged at 320 x g, 4 º C for 5 min. The supernatant was discarded, and the pellet was resuspended in RBC buffer. Cells were briefly incubated, FACS buffer was added, and cells were centrifuged. The supernatant was discarded, and cells were resuspended in 1 mL of FACS buffer for staining.

Spleen samples were ground into single-cell suspension using tweezers and mesh to disrupt the capsid and dissociate the cells in FACS buffer. Samples were homogenized, centrifuged at 320 x g, 4 ºC for 5 min, the supernatant discarded, and the pellet was resuspended in RBC buffer. Cells were briefly incubated, FACS buffer was added, and cells were centrifuged. The supernatant was discarded, and cells were resuspended in 3 mL FACS buffer for staining. Cells were filtered through a 40 μm nylon filter before staining.

All samples collected for flow cytometry staining were kept on ice from the moment of dissection until acquisition on a cytometer unless otherwise specified in the protocol.

3.3.2.3. Flow cytometry staining

For immunophenotyping of immune cell populations, processed samples of blood, peritoneal cavity, lung, and spleen were plated into 96 well plates at an adequate density. Cell suspensions were centrifuged at 320 x g, 4 ºC for 5 min, supernatant was discarded, and pellets were resuspended in TruStain Fc block reagent, diluted in 1 x PBS. Cells were incubated for 25 min on ice. Then washed with 1 x PBS, and centrifuged, the supernatant was discarded, and pellets were resuspended in the appropriate antibody mixes diluted in 1 x PBS and incubated for 25 min on ice in the dark. Antibodies used are listed in the table below. After incubation, cells were washed with FACS buffer, centrifuged, the supernatant was discarded, and cells were resuspended in eBioscience™ Foxp3/Transcription Factor Staining Buffer kit following manufacturers' instructions for fixation.

Samples were acquired up to 48 h after staining and fixation on a Cytek Aurora spectral cytometer (equipped with four lasers (405nm, 488 nm, 561 nm, 640 nm), two SSC detectors, and 48 fluorescence detectors). Unmixing was performed with autofluorescence extractions using unstained controls of the corresponding time point and organ that showed the highest autofluorescence profile. Zombie Live-Dead single colors were always of the respective organs. Results were analyzed using FlowJo software (version 10.10.0).

Table 1 - Antibodies and reagents used for flow cytometry stainings.

Epitope	Clone	Fluorophore	Manufacturer	Catalogue Number
CD4	GK1.5	PE/Cyanine7	BioLegend	100422
CD8a	53-6.7	PE/Cyanine5	BioLegend	100710
CD19	6D5	Brilliant Violet 711	BioLegend	115555
CD69	H12F3	FITC	IGC	
CD62L	MEL-14	PE	eBioscience	12-0621-85
CD3	145.2C11	FITC	IGC	
CD8	YTS169.4	FITC	IGC	

CD90.2	30-H12	AlexaFluor647	IGC	
CD11b	M1/70	PerCP	BioLegend	101229
Ly6G	1AB	PE	BD Pharmingen	551461
CD64	X54-5/7.1	PE/Dazzle594	BioLegend	139320
I-ab	AF6-120.1	PE/Cyanine7	BioLegend	116419
I-ab	KH74	Biotin	A Becton Dickinson Co.	55607
Ly6C	HK1.4	APC	eBiosience	17-5932-82
CD45	30-F11	APC-eFluor780	eBioscience	47-0451-82
Siglec-F	E50-2440	APC-R700	BD Horizon	565183
CD11c	N418	Brilliant Violet 605	BioLegend	117334
F4/80	BM8	Brilliant Violet 711	BioLegend	123147
CD1d (PBS-57)	57001	PE	NIH tetramer core	
CD1d (unloaded)	57002	PE	NIH tetramer core	
CD45b	DX5	PE/Cyanine7	eBioscience	25-5971-82
NK-1.1	PK136	Brilliant Violet 605	BioLegend	108739
Streptavidin		PE/Cyanine5	IGC	
Zombie aqua Fixable viability kit			BioLegend	423102
F4/80	BM8	FITC	BioLegend	123107
F4/80	BM8	AlexaFluor647	BioLegend	123122
TruStain (Fc block)		Unconjugated	BioLegend	101320

3.3.2.4. Data modelling and statistical analysis

All data was analyzed using R studio software. For visualization, the ggplot2 and ggpubr packages were used.

For flow cytometry, results in percentages were analyzed by linear regression in logit-transformed data, whilst absolute count results were analyzed by a negative beta-binomial model to determine the effect of treatment across the different time points independent of experiment.

3.4. Results

3.4.1. Mild mitochondrial stress modulates cellular dynamics during infection

As demonstrated in Chapter 2, data gathered in this work suggests that phenformin-induced mild mitochondrial stress confers protection to septic mice by disease tolerance and resistance mechanisms. To further understand these mechanisms and the cell populations involved, we used flow cytometry to characterize critical immune cell populations of the innate and adaptive arm in different organs. Here, we have decided to characterize the immune system in the blood as the communicating vessel between different tissues; the spleen as a fundamental organ for the initiation, modulation, and maintenance of immune responses; the peritoneal cavity since this specific model of sepsis is induced by intraperitoneal injection and the treatment is administered via this route as well; and finally the lung, as a critical organ in the prognosis of sepsis patient due to its susceptibility to multiorgan failure[37].

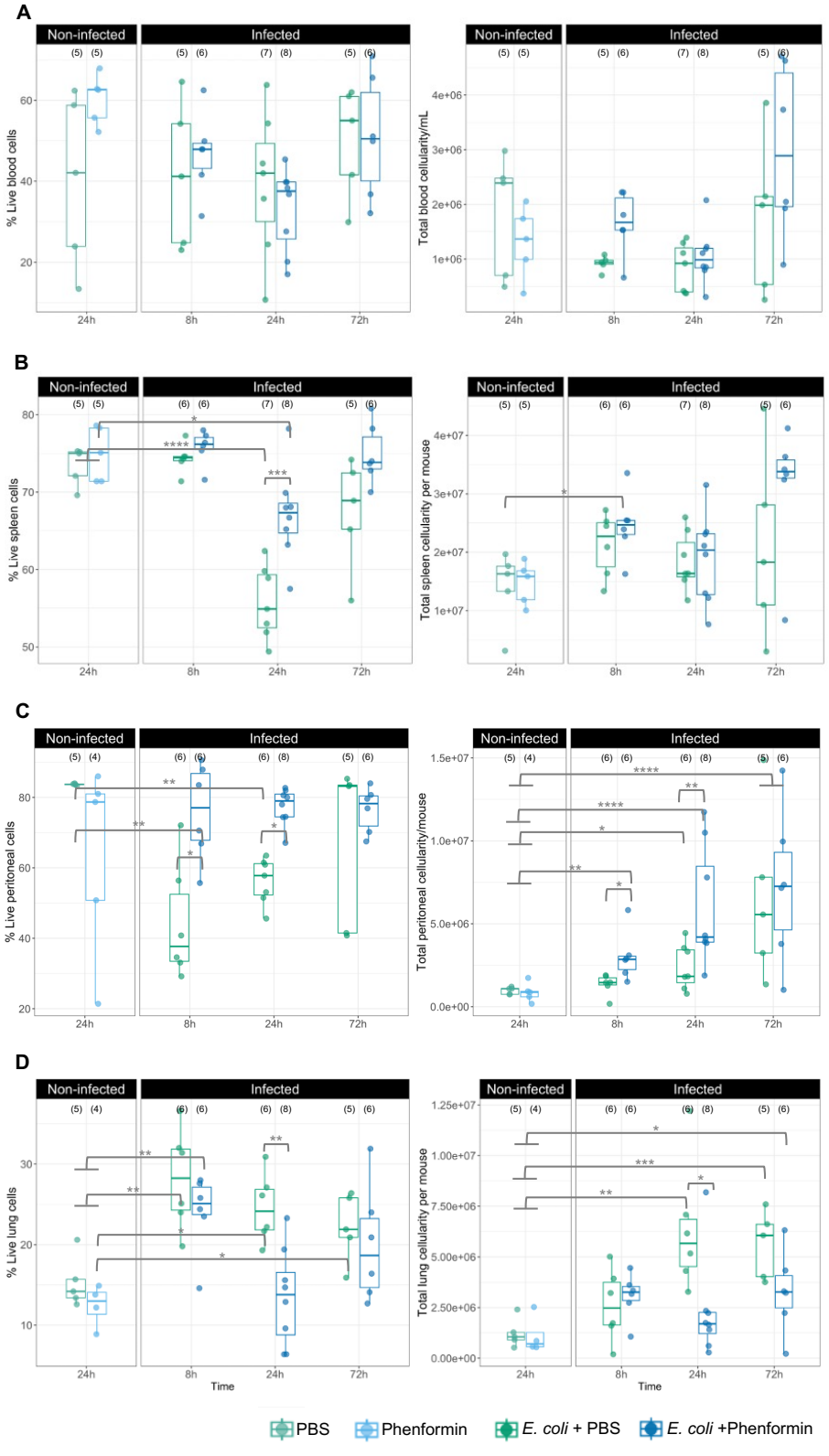
A
Non-infected
Infected
% Live blood cells
Total blood cellularity/mL
B
% Live spleen cells
Total spleen cellularity per mouse
C
% Live peritoneal cells
Total peritoneal cellularity/mouse
D
% Live lung cells
Total lung cellularity per mouse
24h
8h
72h
Time
PBS
Phenformin
E. coli + PBS
E. coli +Phenformin

Figure 3. 1 - Phenformin impacts viability and total cellularity across tissues.

Quantification of percentages of live cells (left side) and total cellularity per organ (right side) in **(A)** blood, **(B)** spleen, **(C)** peritoneum and **(D)** lung of noninfected and infected male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at different time points**. (A-D)** represent pooled data of two independent experiments, individual mice measured are represented by points, and box plots indicate quartiles and mean. **Statistical analysis** was done using R; percentages were analyzed by linear regression in logit-transformed data and counts by negative beta-binomial model to determine the effect of treatment across the different time points independent of experiment. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

In homeostasis, phenformin treatment does not appear to significantly alter viability or total cellularity counts in any of the organs. Both measures are impacted upon infection, and phenformin and vehicle-treated animals present significant differences, as shown in Figure 3.1. In the blood (Figure 3.1(A)), results show some variability between conditions and time points; however, these are not significant. The more pronounced differences can be observed within the other analyzed tissues. Specifically, in the spleen (Figure 3.1 (B)), viable cell percentages significantly drop at 24 h post-infection; however, in phenformin-treated animals, this reduction is not as pronounced, and phenformin-treated infected mice are significantly different from non-treated infected mice at 8 and 24 h post-injection. Furthermore, phenformin-treated animals show a significant increase in spleen cellularity when compared to PBS non-infected mice at 8 h post-infection and injection.

In the peritoneum (Figure 3.1 (C)), infection greatly impacts cells with a striking decrease of viability at 8 h. Interestingly, this only occurs in infected non-treated animals. Phenformin-treated animals do not show any reduction in peritoneal cell viability. Moreover, cellularity counts are elevated with infection in the peritoneum, and phenformin-treated animals show significantly higher cellularity counts when compared to other groups already at 8 h post-infection and treatment, with the highest increase compared to other groups at 24 h post-infection and treatment. Lastly, in the lung (Figure 3.1 (D)), both percentages of viable cells and total cellularity counts increase with infection. However, phenformin-treated animals show smaller deviations from a homeostasis phenotype, presenting lower increases in viable and cell counts.

This fundamental difference in viability and cell numbers due to the treatment of septic mice is, by itself, an interesting indication that phenformin is modulating the immune system and affecting cell recruitment. Immunophenotyping results will be discussed as either the percentage of viable cells or percentages of a parent population of interest. This strategy will allow us to compare population dynamics independently of the impact of phenformin on viability and cellularity.

3.4.2. Organ-specific innate immune cell recruitment is promoted by phenformin treatment of septic mice

Neutrophils are classically known as the first responders during an infection[38]. In fact, our data shows that upon infection with *E. coli*, there is a general increase in neutrophil populations across target tissues and circulating blood, while the spleen shows a reduction, possibly due to the efflux of these cells towards infection sites.

Our data shows that this recruitment is strikingly increased at earlier time points by phenformin to selective tissues. This is very noticeable in the blood and in the peritoneal cavity (Figure 3.2 (A-B) and (E-F) respectively). Already 8 h post-infection, neutrophils have significantly higher percentages in the peritoneal cavity in treated mice. The increase in neutrophil population recruitment is reduced in both groups after 24 h. Spleen neutrophils reduce with infection. This is possibly due to the efflux of these cells towards infection sites. This is mostly pronounced at 8 h post-infection and only significant in non-treated infected mice, while phenformin-treated mice have almost similar neutrophil levels as non-infected mice. Interestingly, the lungs of phenformin-treated mice have less neutrophil infiltration (Figure 3.2 (G-H)).

Monocytes and macrophages are also important in the progression of sepsis and the immune response to infections. These cell subsets are key players in maintaining the recruitment of immune cells and fine-tuning the immune response by releasing cytokines that will modulate the immune system toward the appropriate profile[39,40]. Thus, we have also characterized these cell populations throughout the tissues.

Our results show a decrease in blood Ly6C(+) monocytes associated with the infection that is more pronounced in phenformin-treated animals up to 24 h, after which treatment appears to promote recovery to homeostatic levels of non-infected mice blood monocytes (Figure 3.3 (A-C)). The expression of Ly6C on monocytes has been demonstrated to distinguish different subsets with distinct functional characteristics. Herein, high expression of Ly6C is associated with a pro-inflammatory profile, and these monocytes play major roles in inflammation, with increased recruitment via CCR2 signaling, whereas Ly6C low expression is a key feature of patrolling monocytes that are crucial for wound healing and anti-inflammatory processes[41,42]. Thus, our results indicating the decrease of these cells in circulation could suggest an increased recruitment of monocytes to infection sites, which appears augmented by phenformin treatment. Additionally, blood macrophages are greatly increased at 8 h post-infection in the phenformin-treated group and remain increased when compared to non-

infected and infected control animals up to 24 h, while vehicle-injected infected mice do not show significant increases in this population until 72 h post-infection (Figure 3.3 (B-C)).

In line with this, spleen macrophages are reduced upon infection. Similarly to neutrophil populations, this could result from the efflux of these cells from the spleen towards sites of infection. Moreover, similar to what is observed with neutrophil populations, phenformin-treated animals show lower reductions and faster recovery to the baseline of non-infected levels already at 24 h (Figure 3.3 (D-F)). Interestingly, red pulp macrophages, a population that is involved in the clearance of old and damaged red blood cells, strikingly increase within 8 h of infection, and this increase is significantly more pronounced with phenformin treatment (Figure 3.3 (E-F)).

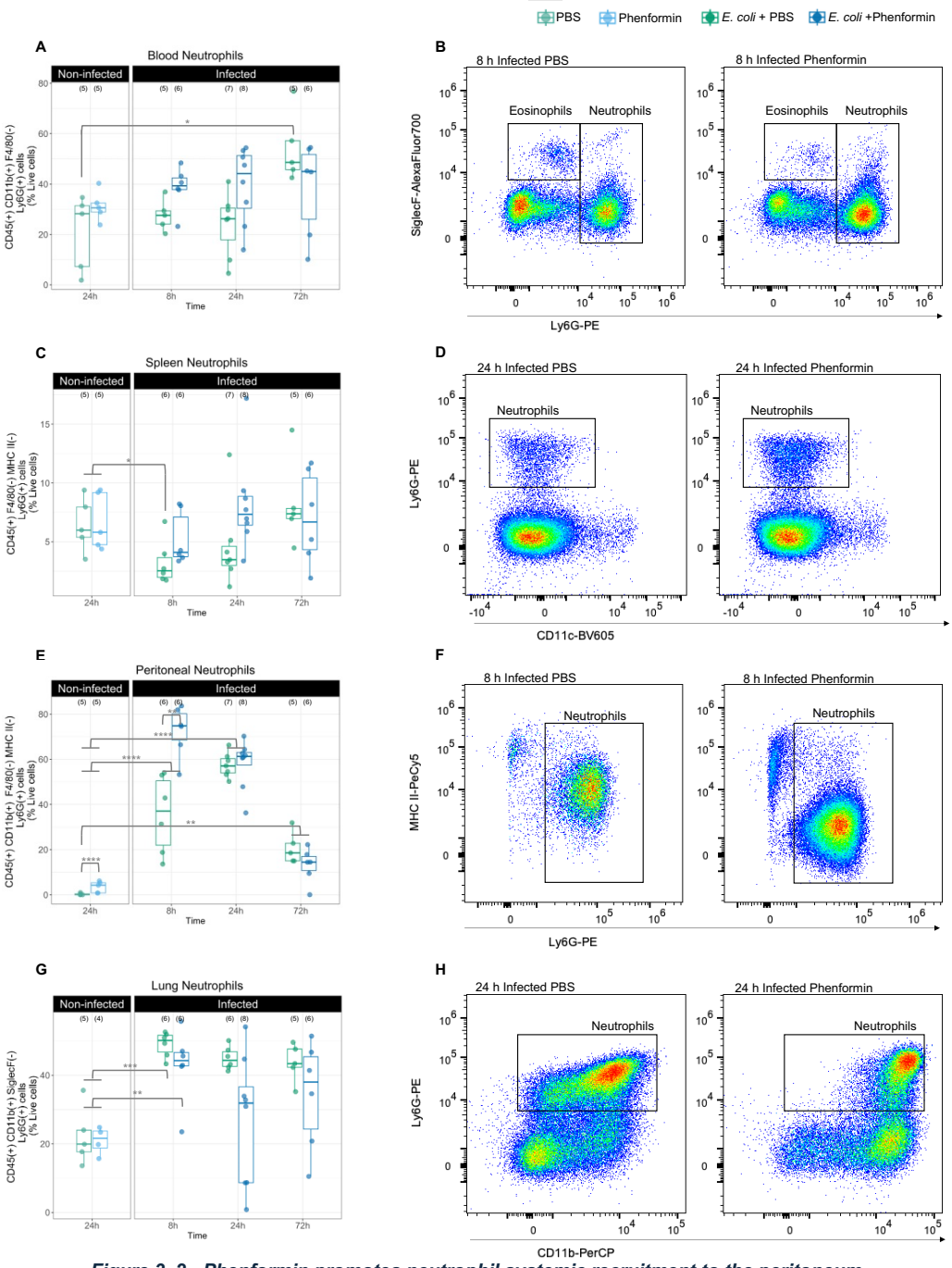

Figure 3. 2 - Phenformin promotes neutrophil systemic recruitment to the peritoneum.

Neutrophil populations in **(A, B)** blood, **(C, D)** spleen, **(E, F)** peritoneal lavage fluid and **(G, H)** lung of noninfected and infected male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A, C, E, G)** Percentages of neutrophils within the live cell population in the indicated organs at different conditions and time points. **(A, C, E, G)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and mean. **(B, D, F, H)** Representative pseudocolor plots of neutrophil populations at the indicated tissues and time points post-infection

and control vehicle solution injection (left side) or with phenformin treatment (right side). Gating strategies for each organ-specific population can be found in Supplementary Figures. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

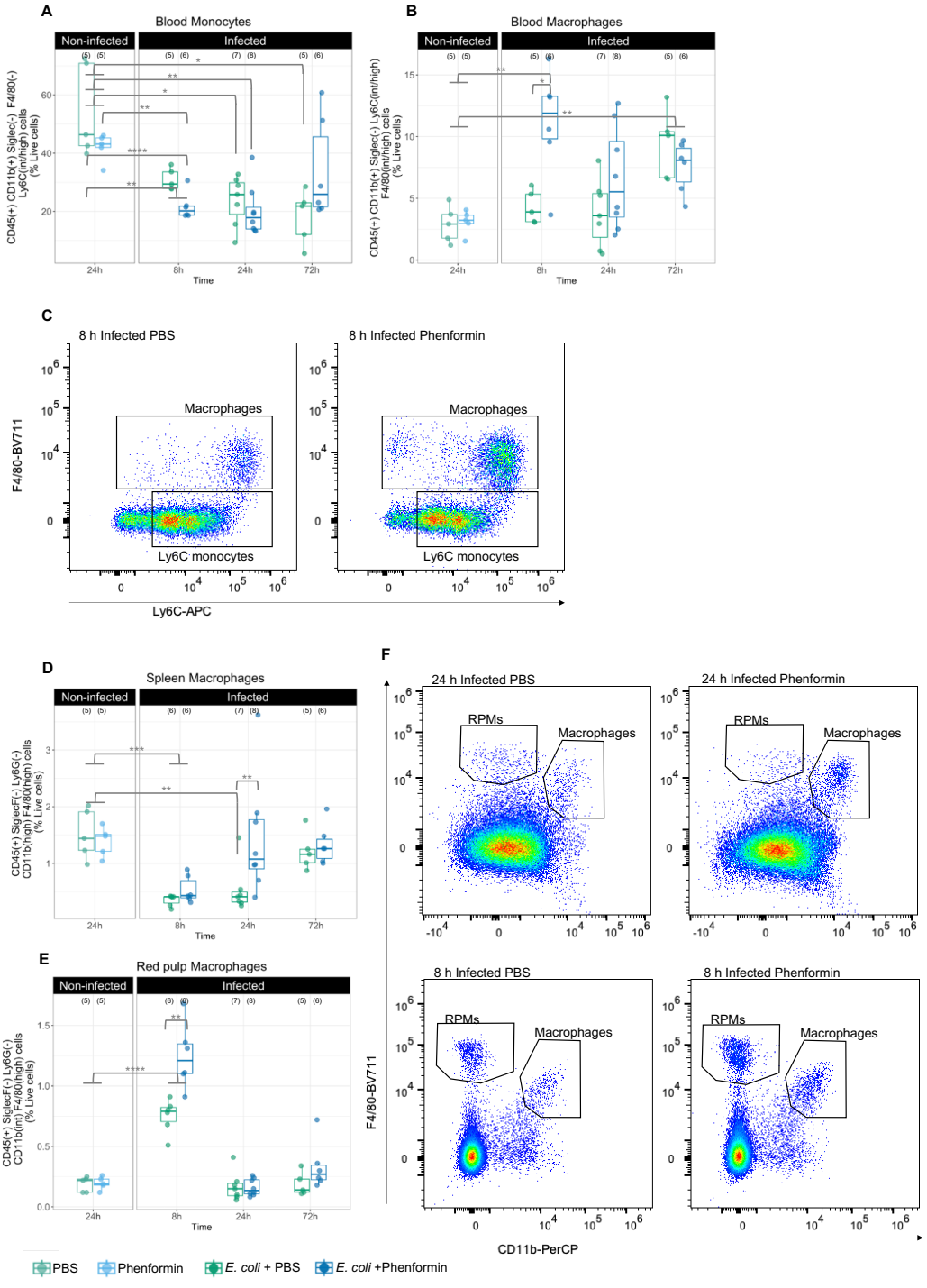

Figure 3. 3 - Macrophage and blood monocyte population homeostasis during infection is enhanced by phenformin.

Macrophage populations in **(A, C)** blood and **(D, E, F)** spleen, and monocytes in **(B, C)** blood of non-infected and infected male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A, B, D, E)** Percentages of macrophages and monocytes within the live cell population at the indicated organs at different conditions and time points. **(A, B, D, E)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and mean. **(C, F)** Representative pseudocolor plots of macrophages and monocytes in infected blood and spleen samples respectively at indicated time points after infection and injection of control vehicle solution (left side) or with phenformin treatment (right side). Gating strategies for each organ-specific population can be found in Supplementary Figures. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Remarkably, macrophages from the peritoneal cavity already show alteration with the administration of phenformin in non-infection conditions. Results in Figure 3.4 show that phenformin intraperitoneal injection significantly reduced the large peritoneal macrophage (LPM) population (a phenomenon previously described as macrophage disappearance reaction[43]) while promoting the income of a population with intermediate expression of the classical macrophage marker F4/80 and low to intermediate expression of MHC II (Figure 3.4 (A)). Considering the work of Vega-Pérez *et al.* and our results shown here, we believe that this population is composed of infiltrating monocytes[44]. With infection, macrophages from the peritoneum are severely compromised. As our results show, in infected mice, both small and large peritoneal macrophage populations were drastically reduced. This is in agreement with previous studies that support the importance of these immune cell populations in peritoneal bacterial clearing[44]. Interestingly, even though phenformin-treated infected animals also suffer a reduction in small and large peritoneal macrophages (SPMs and LPMs, respectively) at 8 and 24 h, monocyte recruitment is promoted by phenformin more efficiently at 24 h. In turn, at 72 h, phenformin-treated animals show increased small and large peritoneal macrophages when compared to their control infected counterparts, suggesting the commitment and differentiation of the infiltrating monocytes towards tissue-resident macrophage profiles, thus promoting faster recovery of these immune cell populations.

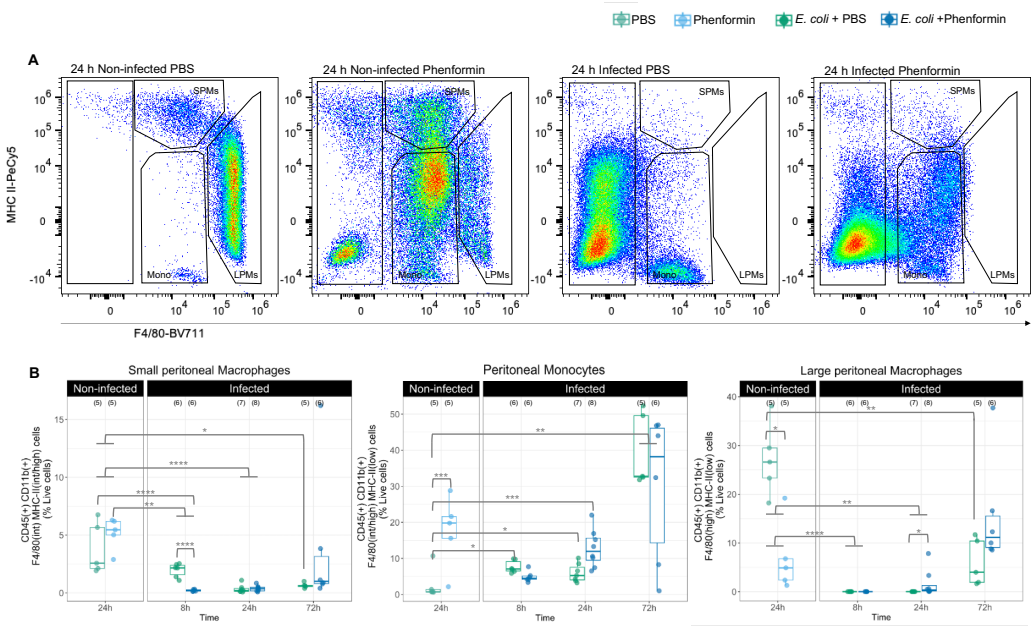

Figure 3. 4 - Mitochondrial stress triggers macrophage disturbance of homeostasis reaction and promotes pre-emptive monocyte recruitment to the peritoneum.

Macrophage populations in the peritoneum of non-infected and infected male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A)** Representative pseudocolor plots of SPMs (Live cells, CD45(+) CD11b(+) F4/80(int) MHC-II(int/high)), monocytes (Live cells, CD45(+) CD11b(+) F4/80(int/high) MHC-II(low/int)) and LPMs (Live cells, CD45(+) CD11b(+) F4/80(high) MHC-II(low)) populations in peritoneal lavage fluid samples (from left to the right side) in non-infected or infected, vehicle control or phenformin-treated at 24h post-injection and when indicated infection. **(B)** Percentages (from left to right side) of small peritoneal macrophages (SPMs)**,** monocytes (mono)**,** and large peritoneal macrophages (LPMs) within live cells in different conditions and time points. **(B)** represents pooled data of two independent experiments, individual mice are represented by points, and box plots indicate quartiles and median. The gating strategy can be found in Supplementary Figures 3.9. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Lung tissue-resident macrophages can broadly be divided into alveolar, interstitial, and monocyte-derived immature macrophages. The latter can ultimately repopulate both alveolar and interstitial niches upon specific cues[40,45,46]. The balance between these macrophage populations is essential for a proper immune response against an insult and restoration of homeostasis.

In this work, we followed the gating strategies of identification for lung tissue-resident macrophages, previously published[47,48]. Alveolar macrophages were identified as described in the publications as CD11b(-) SiglecF(+) CD11c(+) CD64(+) cells. However, inflammation and phenformin treatment result in significant changes in the expression of specific markers used for the identification of interstitial and monocyte-derived populations (most importantly

affected by MHC II modulation as observable in the representative pseudocolor plot in Figure 3.5 (C)), because of this, we adapted our gating strategy. Thus populations were classified as: interstitial macrophages defined as CD11b(high) MHC II(high) F4/80(+) CD64(+) cells; (monocyte-derived) immature macrophage-like cells as CD11b(high) MHC II (low/intermediate) F4/80(high) CD64(high) cells.

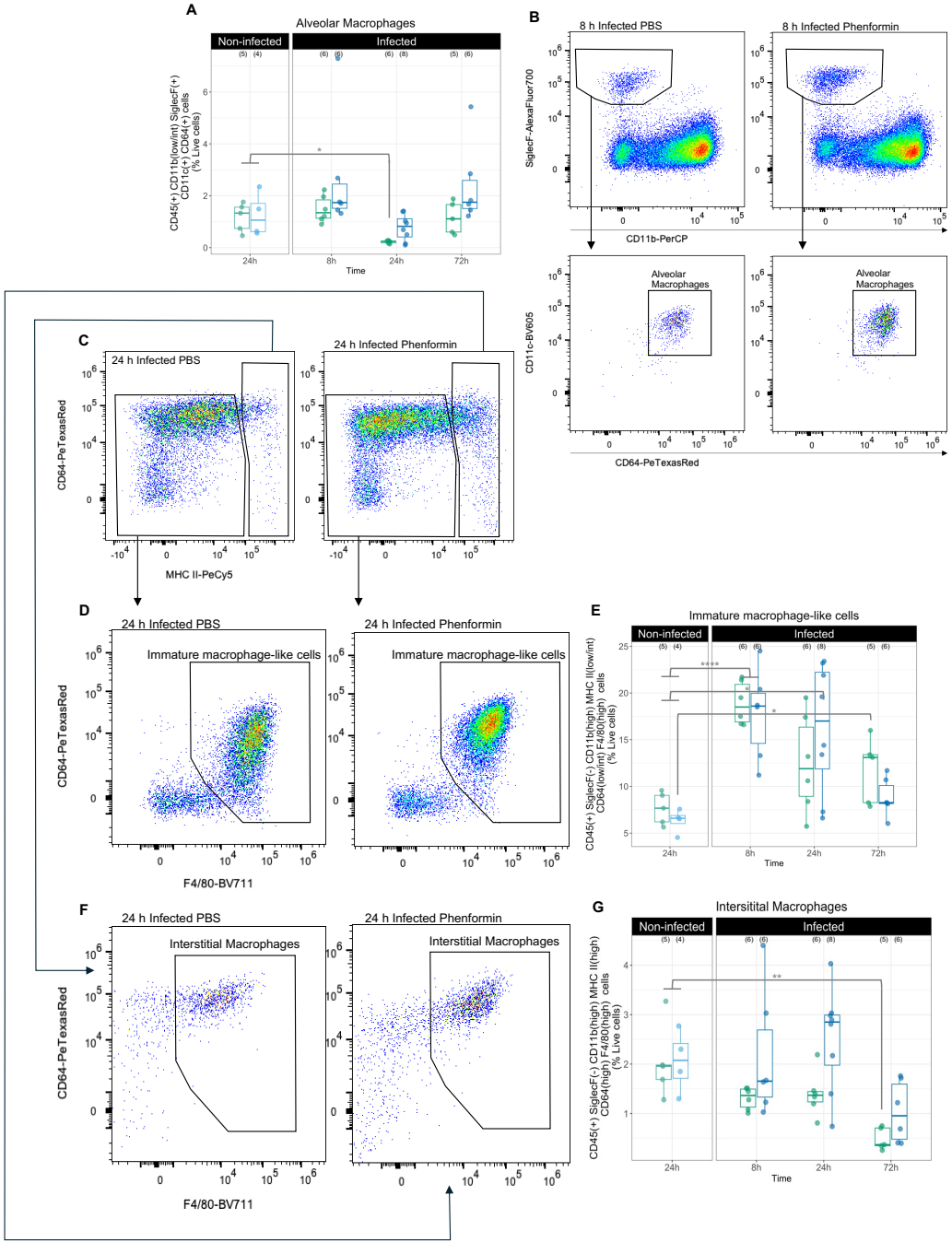

Figure 3. 5 - Phenformin modulates immune cell recruitment to the lung during infection.

Macrophage populations in lung of non-infected and infected male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A)** Percentages of alveolar macrophages within the live cell population, **(B)** Representative pseudocolor plots of alveolar macrophages in infected lung samples 8 h after infection and injection of control vehicle solution (left side) or with phenformin treatment (right side). **(C, D, F)** Representative pseudocolor plots of **(C)** CD11b(high) populations, **(D)**

immature macrophage-like cells (MHC II(low/int) CD11b(high)), **(F)** interstitial macrophages (MHC II(high) CD11b(high)). **(E, G)** Quantification of immature macrophage-life lung cells (E) and interstitial macrophages (G) in percentages within the live cell population in lung at different conditions and time points**. (A, E, G)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and median. Gating strategy can be found in Supplementary Figure 3.6. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Alveolar lung macrophages are key players in lung immune surveillance and the initiation of primary inflammatory responses. Although this cell population does not present a great variation, phenformin-treated mice generally show similar percentages of these cells to homeostatic conditions, while vehicle-treated infected mice show a significant reduction of alveolar macrophages at 24 h post-infection in their lungs (Figure 3.5 (A-B)).

In line with this, interstitial macrophages also show a significant reduction 72 h after infection in non-treated animals (Figure 3.5 (F-G)). After infection, we observe a great influx of immature macrophage-like cells in both treated and non-treated animals (Figure 3.5 (D-E)). Although this recruitment is more pronounced in phenformin-treated animals within the first 24 h, treated animals manage to normalize immature macrophage-like cell percentages to homeostatic levels at 72 h, while non-treated infected animals still show a significant increase in cells. While monocyte recruitment is a key event during inflammation and fundamental for pathogen clearance, prolonged or exacerbated recruitment of this cell population has also been associated with increased tissue damage and contributes significantly to immunopathology[49]. Thus, phenformins' ability to control monocyte recruitment earlier on during infection might exert a beneficial effect through disease tolerance.

3.4.3. Phenformin-modulated lymphoid cell migration

Lymphocytes also play a fundamental role in maintaining an appropriate immune response. In many cases, an erroneous lymphocyte-driven immune response culminates in exacerbated reactions and extensive tissue damage[50]. In Supplementary Figure 3.1, we show quantifications of CD4, CD8, B lymphocytes, NKT, and NK cells across tissues and during the time course of infection.

With infection, we have observed a reduction of CD8 and B cell populations in the blood, peritoneum, and lung, which is more pronounced in phenformin-treated animals. While in the spleen B cells increase, this increase is less pronounced with phenformin. These effects are mostly observable at 8 and 24 h. On the other hand, natural killer cells show an opposing phenotype to lymphocytes, with a reduction in the spleen and an increase in the blood.

Notwithstanding, phenformin-treated animals show a lower increment of circulating NK cells when compared to control-infected animals.

These results could overall indicate that phenformin is modulating lymphoid immune cell migration during infection. Most strikingly, with the decrease of T lymphocytes within the organism combined with the increased populations in the spleen. On the other hand, natural killer cells, which cannot be categorized as lymphocytes but also have distinctive characteristics from innate cells, appear to increase in circulation. To further understand what the fluctuations in these cell types could implicate, we have characterized these populations and their activation status by expression of early activation marker, homing marker, and metabolic gatekeeper CD69.

3.4.4. Adaptive immune cell activation is controlled by phenformin treatment of septic mice

In homeostasis, lymphocytes do not express CD69. In line with this, we do not observe a difference in the expression of this marker with the treatment of non-infected mice with phenformin. However, upon infection with *E. coli,* CD69 is highly upregulated in all lymphocyte, NKT, and NK cell populations.

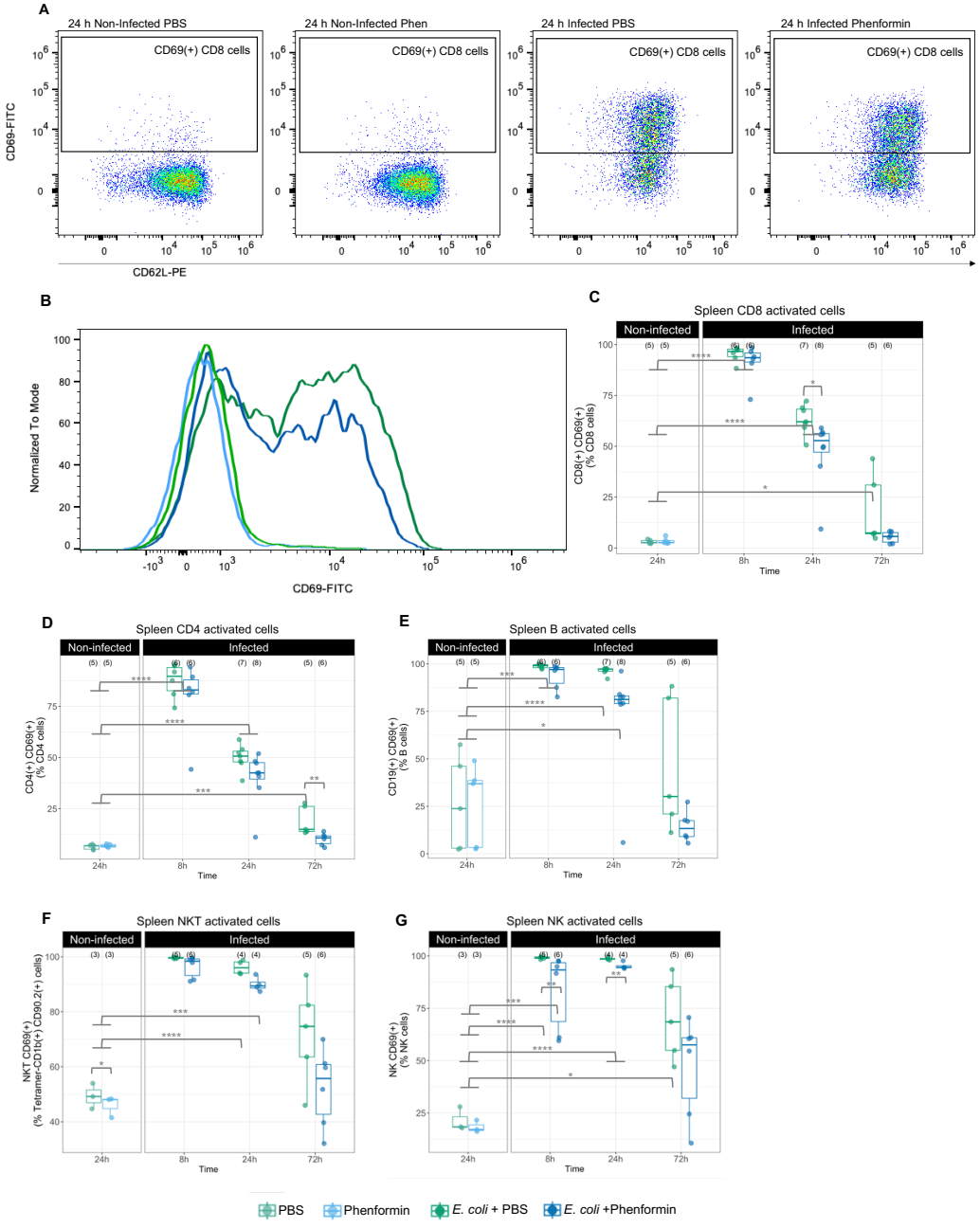

Figure 3. 6 - Phenformin reduces lymphocyte and NK cell infection-driven activation.

Percentage of CD69(+) in different lymphocyte, NKT and NK subsets of the spleen non-infected and infected male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at 8, 24, 72 h. **(A)** Pseudocolor plot of data from CD62L expression on the x axis and CD69 on the y axis of total CD8 T cells at 24h of non-infected PBS, non-infected Phen, infected PBS, infected Phen animals (from left to right). **(B)** Histogram display overlay, normalized to mode, of representative plots for phenformin and vehicle (PBS) mice spleen cells at 24 h post-injection and infection (or control non-infected) within total CD8 T cells. Activated **(C)** CD8, **(D)** CD4, **(E)** B, **(F)** NKT, **(G)** NK cells in percentages within respective parent population

percentage in the spleen at different conditions and time points**. (C, D, E, F, G)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and median. Gating strategies can be found in Supplementary Figures 3.3 and 3.4. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Our results show that treatment of infected mice with phenformin reduces the expression of the CD69 surface marker in all lymphocyte populations. This phenotype is also observable in NKT and NK cells, which cannot be classified as adaptive immune cells but express the same activation marker on their surface. Figure 3.6 (C-G) shows the quantification of spleen populations, a representative pseudocolor plot for spleen CD8 cells at 24 h post-infection (Figure 3.6 (A)), and a histogram overlay of CD69 expression on CD8 cells of non-infected and infected (Figure 3.6 (B)), vehicle and phenformin-treated mice. Quantifications for blood, lung, and peritoneal cavity can be found in Supplementary Figure 3.2.

Albeit conserved across populations and tissues, this phenformin-driven CD69 reduction occurs at different degrees and time points depending on the cell population. NK cells are highly and rapidly responsive to insults, interestingly, reduction of CD69 in these cells occurs very early on, already at 8 h after infection and treatment. CD8 T cells are the most aggressive and the first responders within T cell populations. CD69 reduction in CD8 cells is more pronounced at 24 h post-infection. On the other hand, CD4 T and B cell responses require longer. CD69 reduction in these cells is more pronounced later, mostly significant at 72 h post-infection and treatment.

Besides being an early activation marker, CD69 has also been extensively described in the literature as a metabolic gatekeeper that can skew CD4 T helper cells into a more regulatory or pro-inflammatory profile[51], and it is involved in CD8 T effector function and NK cell degranulation and cytotoxicity[52–54]. Additionally, it is involved in homing or egression to lymphoid organs for immune response development of lymphocytes and NK cells[55].

3.5. Discussion

Sepsis is a multifactorial inflammatory condition that results in organ failure derived from extensive tissue damage. This condition can be triggered by an infection caused by various pathogens but is characterized by exacerbated host responses[56]. Because of the nature of the tissue damage leading to organ failure and considering the results from Chapter 2, we have characterized the immune cell populations across tissues in non-infected and infected animals with and without phenformin treatment. This will allow us to understand whether

phenformin can modulate immune dynamics and possibly identify key immune cell populations involved in disease tolerance and resistance mechanisms.

Our results show an impact of phenformin on cell viability and organ total cellularity that is strikingly visible across all tissues during infection. Herein, phenformin-treated animals tend to show more similarity with non-infected animals in viability percentages, demonstrating a faster recovery from the burden of the infection or a lower impact imposed by the infection. Interestingly, in total cellularity counts, phenformin-treated animals have higher numbers when compared to other groups of animals. This suggests an increased recruitment or differentiation capacity, which will be subject to study in Chapter 4.

Our results suggest an organ- and cell-type-specific recruitment of specific immune cell populations. In detail, we can observe a targeted recruitment of neutrophils towards the site infection initiation, the peritoneum. Furthermore, this appears to result from systemic responses, as suggested by the efflux of neutrophil and macrophage populations from secondary lymphoid organs. This is indicated by the reduction of these cell populations from the spleen towards the blood at 8 h, culminating with an increase in the peritoneum from 8 h until later.

Interestingly, phenformin-induced immune recruitment is biased towards the peritoneum, with a different dynamic landscape in the lung. In fact, in the lung, phenformin appears to control neutrophil infection-derived cell influx. This organ-specific difference might be beneficial since neutrophils have been described as key players in the exacerbation of lung organ damage[40,45,47,48,57]. Furthermore, the innate immune populational shifts associated with phenformin in the lung of treated-infected mice indicate a favoring of tissue protection, as evidenced by the lower impact of infection on alveolar macrophages, faster reduction of the percentage of immature macrophage-like cells and return to homeostatic levels of interstitial macrophages driven by phenformin. Considering the described fundamental role of incoming monocytes, monocyte-derived macrophages, and neutrophils in the progression of lung injury, we can speculate that controlling the influx of these cell types might prevent extensive lung injury[48,57].

Of particular interest is the impact of phenformin in peritoneal cavity macrophage populations. We have observed that administration of phenformin in homeostasis appears to lead to macrophage population reductions. This phenomenon resembles the described macrophage disappearance reaction (MDR), or most recently renamed Macrophage Disturbance of Homeostasis Reaction (MDHR)[43,58,59]. In parallel, a population of cells that is acquiring a macrophage-like profile is recruited. Due to the lack of proper identification markers, we can

only speculate, considering what was described in other studies, that these might be the described Ly6C(high) monocytes shown in Vega-Peréz and colleagues work[44] to be recruited after MDR for *E. coli* clearing. Similarly to what was described in this same study, at 8 h post-infection both control and phenformin-treated mice do not have tissue-resident macrophages, but later, at 24 h, phenformin-treated animals have an increase in monocytes, possibly indicating from recruitment and differentiation of Ly6C(high) monocytes into tissue-resident macrophages. In fact, a study using a reporter mouse line for granulocyte-monocyte progenitors (GMPs), showed that replenishment of LPMs can occur through Ly6C(high) monocyte differentiation via bone-marrow myelopoiesis[59]. Our results ultimately show an accelerated recovery of SPM and LPM populations noticeable at 72 h after infection and treatment with phenformin. Altogether, these data suggest that phenformin might be leading to MDHR even in the absence of infection and, in this way, triggering an earlier stimulation of myelopoiesis. Ultimately, in an infection context, having this phenformin-driven priming might be protective, as it will harness mechanisms that promote cell recruitment during an infection context.

Remarkably we see a different phenotype in lymphoid cell populations. Here, instead of an increase of these cells at infection sites, we can observe a decrease of T and B populations in the blood, peritoneum, and lung, indicating a possible phenformin-driven homing to the spleen. On the other hand, in terms of recruitment, NK cells show a more similar pattern to innate cells, with an increased recruitment to the site of infection. This increased recruitment appears, however, controlled by phenformin within the first 8 h, with results showing different degrees of increases of NK cells in the peritoneum as compared to control infected animals. Interestingly this is at latter time points followed by a phenformin-driven boost of NK cell recruitment at 24 h, surpassing the recruitment of NK cells in control infected animals. Despite different impacts on the migration dynamics of these cell populations, phenformin appears to have a conserved functional effect on T, B NKT, and NK cells, the reduction of the surface expression of CD69.

CD69 was first identified as an early activation marker of lymphocyte populations. It is a type II C-lectin receptor, and the gene is located in the natural killer gene cluster, presenting binding sites for inducible transcription factors with important roles in the immune response, including nuclear-factor (NF)-kB, erythroblast transformation-specific related gene-1 (ERG-1) and activator protein 1 (AP-1). Because of this, CD69 is also a key player in modulating and sustaining T cell responses, being involved in T cell proliferation[51]. Albeit classically described for CD4 T cells, CD69 also plays a role in CD8 T cells and NK cells[54,60]. In fact, in NK cells, CD69 was shown to activate the cytolytic machinery of this cell population. The ability of CD69

to modulate the activation is commonly shared by all hematopoietic lineage cells and relies on Ca^{2+} mobilization[54,61]. More recently, studies have brought evidence to light that CD69 functions surpass activation and proliferation of hematopoietic linage cells, having been found responsible for cell egress and lymphocyte retention in lymphoid tissues during infection[55], lymphocyte differentiation, metabolic rewiring and modulation of functional capacities[51,62,63]. In fact, many fields have taken an interest in modulating CD69 expression, including immunotherapies for cancer or autoimmune disease management[53,64].

Because of the many possible roles CD69 can have and the generalized phenotype of reduction (albeit at different levels and time points) we have observed in our data, we can speculate that the reduction of this marker in so many cell populations could be associated with reduced tissue damage in our model of sepsis treatment. Most importantly, we should consider that the time points at which we see CD69 reduction with phenformin treatment coincide with the time points at which we see lower tissue damage scores without pathogen reduction.

An important consideration for the results reported regarding lymphocytes, that should be subject to validation, is that the gating strategies employed to identify T cells resorted only to the identification by the cell surface marker CD4 for T helper cells, and CD8 for T cytotoxic cells. The staining protocol did not include a CD3 marker, nor a dump channel for dendritic cells (DCs), NK, or NKT cell exclusion. In humans, it has been described that CD8 and CD4 markers can be expressed by other immune cells, such as DCs and NK cells. Furthermore, these expressions were also shown to be influenced by challenges or alterations, such as exercise or infections[65]. Although this is not common in mice, it cannot be discarded that during infection, this could change[66]. Thus, the accuracy of these results should be subject to validation.

Notwithstanding this caveat, together these data indicate that phenformin has an immunomodulatory capacity that might be cell- and organ-specific. Considering the different default program biases and response kinetics of innate and adaptive immune cells, it is not surprising that inhibition of complex I by administration of phenformin could elicit opposing effects. While both innate and adaptive immune cells rely on a switch to glycolysis to fulfill their energetic demands during their functional responses to a challenge, innate cell responses, such as macrophages and neutrophils, are characteristically rapid and seemingly unspecific, whereas adaptive immune cells' function involves processes of migration, proliferation, and affinity maturation which ultimately result in longer response times[29,32,67–71]. Thus, it becomes reasonable to speculate that the same mild inhibition of complex I could distinctly impact these cell subsets, given their effector function response kinetics. NK cells are more complicated to

classify in this sense, as they rely on both OXPHOS and glycolysis for their cytotoxic properties[72–74]. In the following chapter, we will focus on dissecting on a mechanistic and functional level the impact of the phenotypic changes we have described so far.

Acknowledgments

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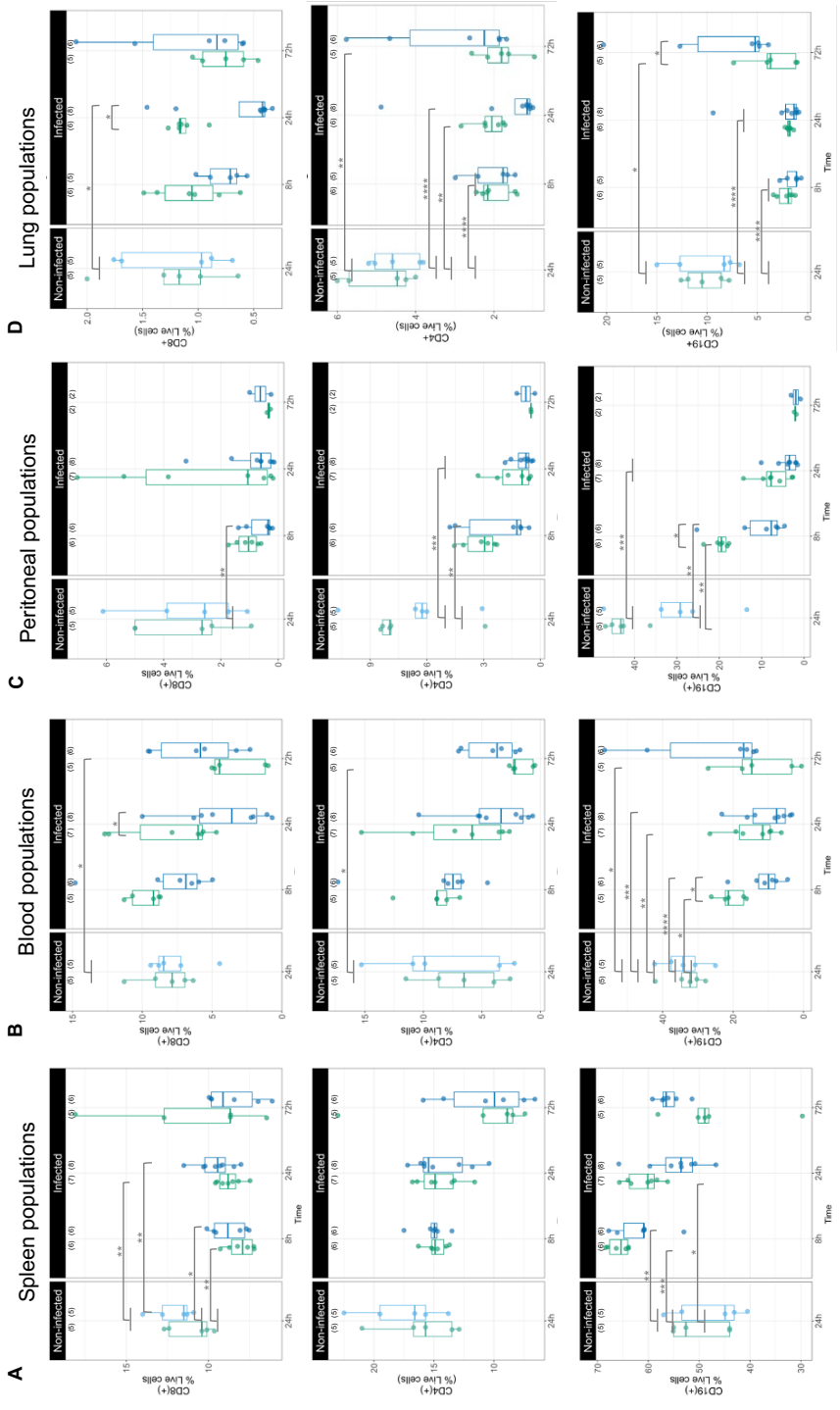
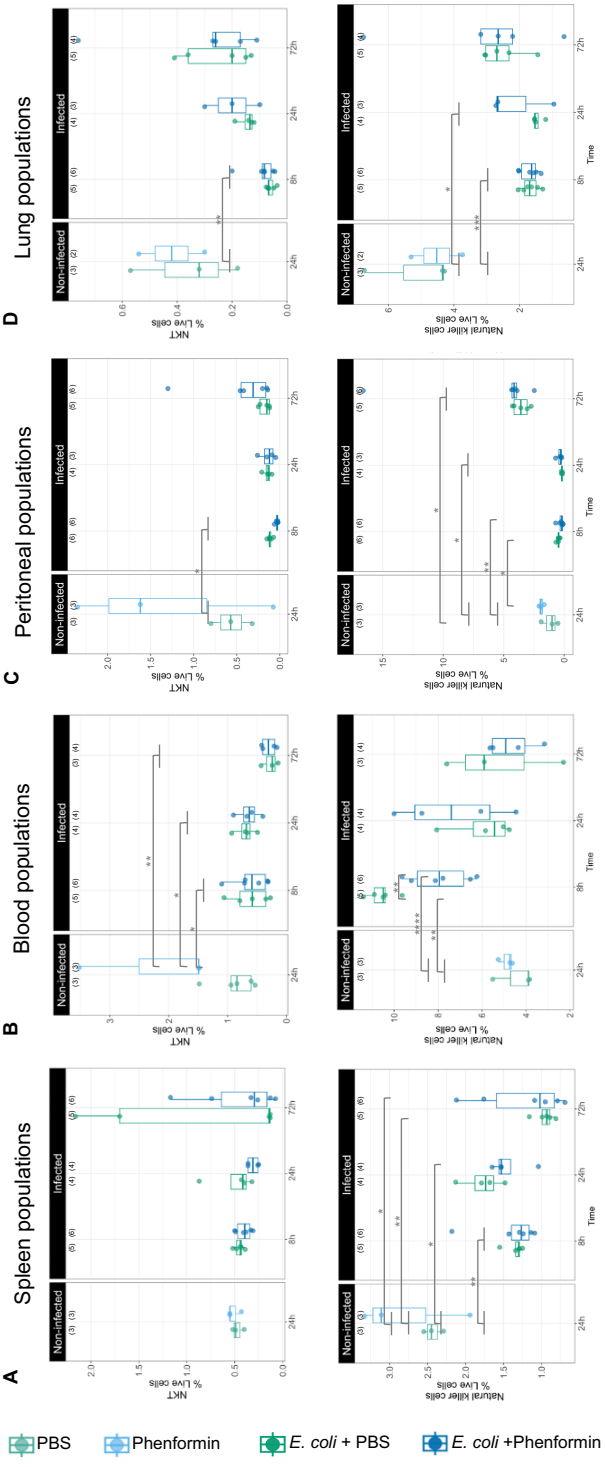
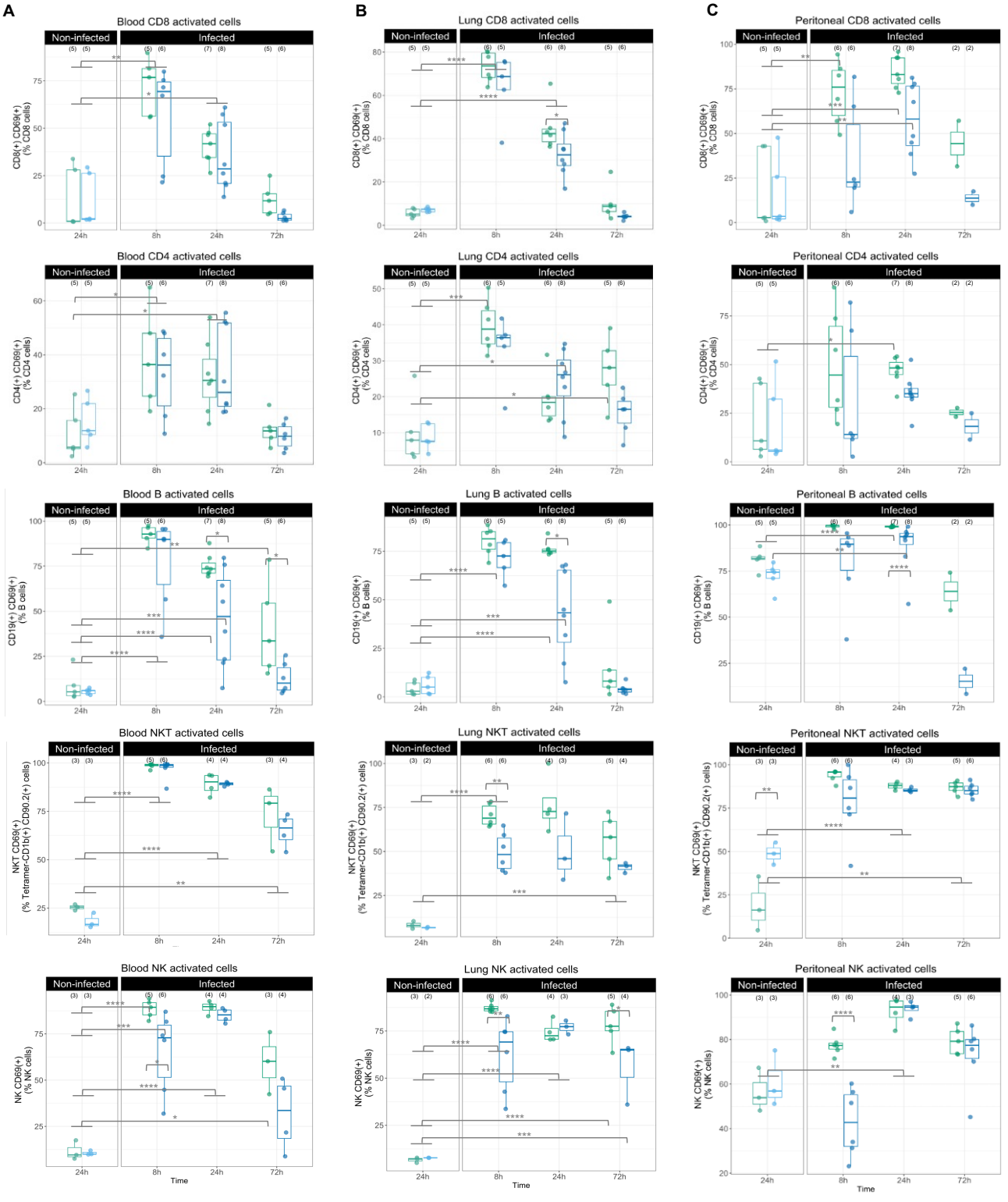
3.7. Supplementary data





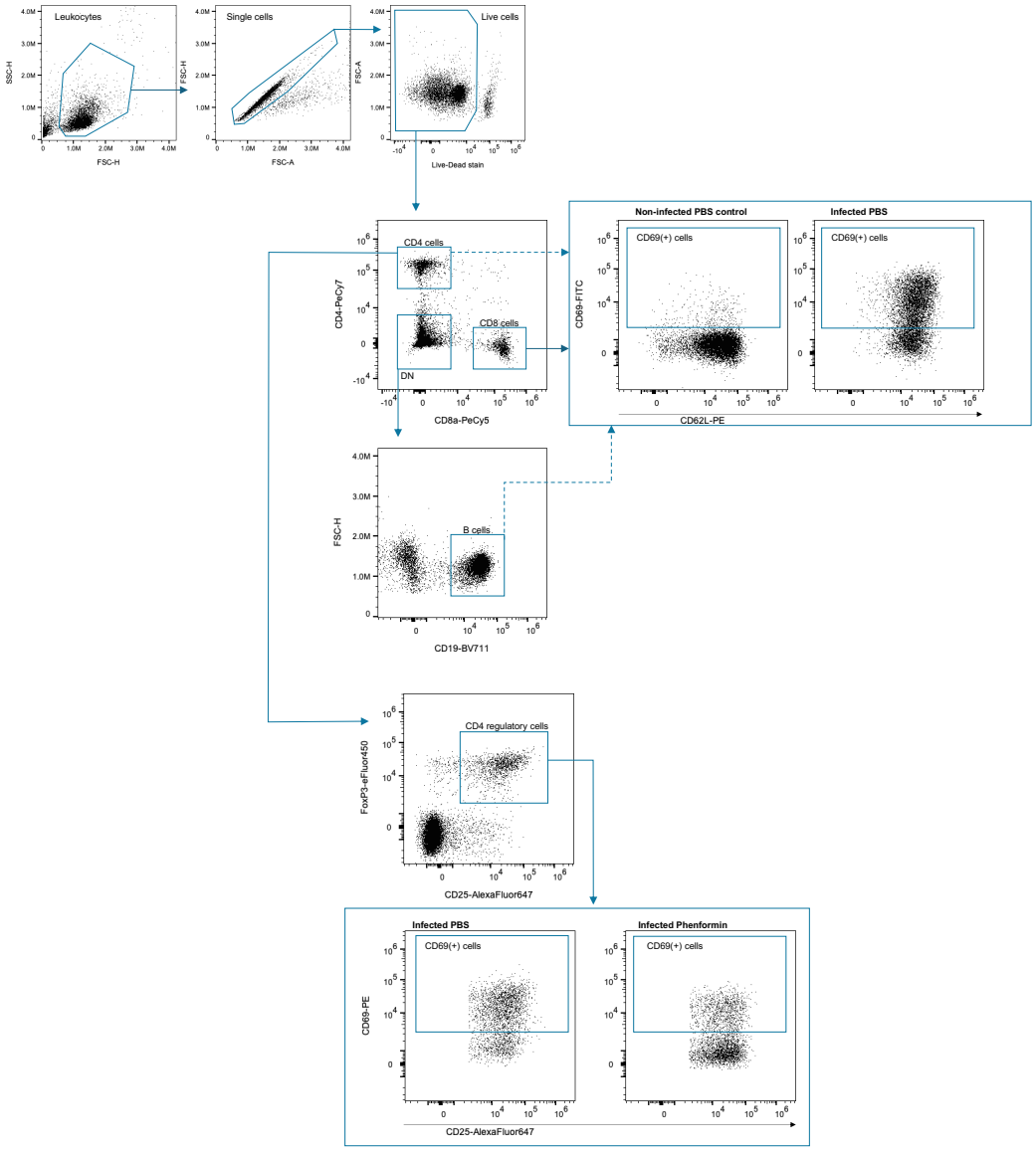
Supplementary Figure 3. 1 - Phenformin modulates lymphocyte and NK cell migration during infection.

Percentage of different lymphocyte, NKT and NK subsets from the **(A)** spleen, **(B)** blood, **(C)** peritoneum and **(D)** lung of non-infected and infected male C57BL/6J mice with 3 - 4 x10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at 8, 24, 72 h. From upper to lower plots, CD8, CD4, B, NKT, NK cells in percentages of live cells across different tissues, conditions and time points. **(A-C)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and median. Gating strategies can be found in Supplementary Figures 3.3 and 3.4. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

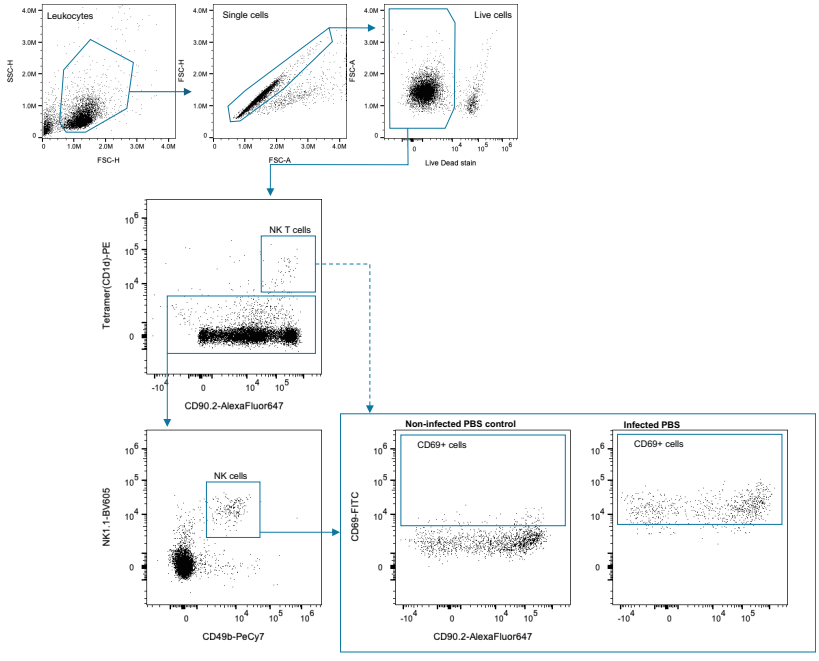

A
B
C
Blood CD8 activated cells
Lung CD8 activated cells
Peritoneal CD8 activated cells
Blood CD4 activated cells
Lung CD4 activated cells
Peritoneal CD4 activated cells
Blood B activated cells
Lung B activated cells
Peritoneal B activated cells
Blood NKT activated cells
Lung NKT activated cells
Peritoneal NKT activated cells
Blood NK activated cells
Lung NK activated cells
Peritoneal NK activated cells
Non-infected
Infected
24h
8h
24h
72h
Time
CD8(+) CD69(+) (% CD8 cells)
CD4(+) CD69(+) (% CD4 cells)
CD19(+) CD69(+) (% B cells)
NKT CD69(+) (% Tetramer-CD1b(+) CD90.2(+) cells)
NK CD69(+) (% NK cells)
PBS
Phenformin
E. coli + PBS
E. coli +Phenformin

Supplementary Figure 3. 2 - Phenformin reduces lymphocyte and NK cell activation during infection across tissues.

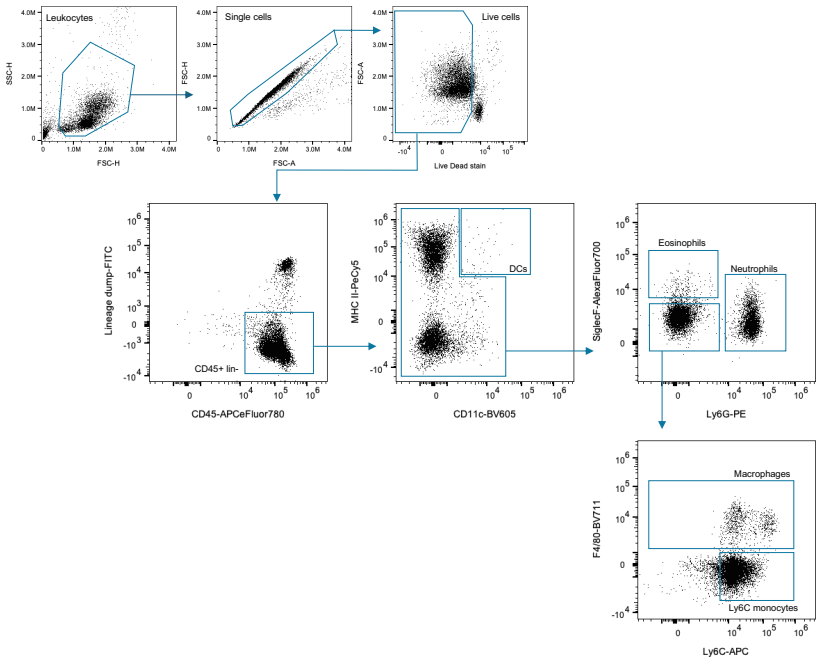
Percentage of CD69(+) in different lymphocyte, NKT and NK subsets from the **(A)** blood, **(B)** lung, and **(C)** peritoneum of non-infected and infected male C57BL/6J mice with 3 - 4 $x10^8$ CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at 24, 72 h. From upper to lower plots, activated CD8**,** CD4, B, NKT, NK cells in percentages within respective parent population across different tissues, conditions and time points**. (A-C)** represent pooled data of two independent experiments, individual mice are represented by points and box plots indicate quartiles and median. Gating strategies can be found in Supplementary Figures 3.3 and 3.4. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).



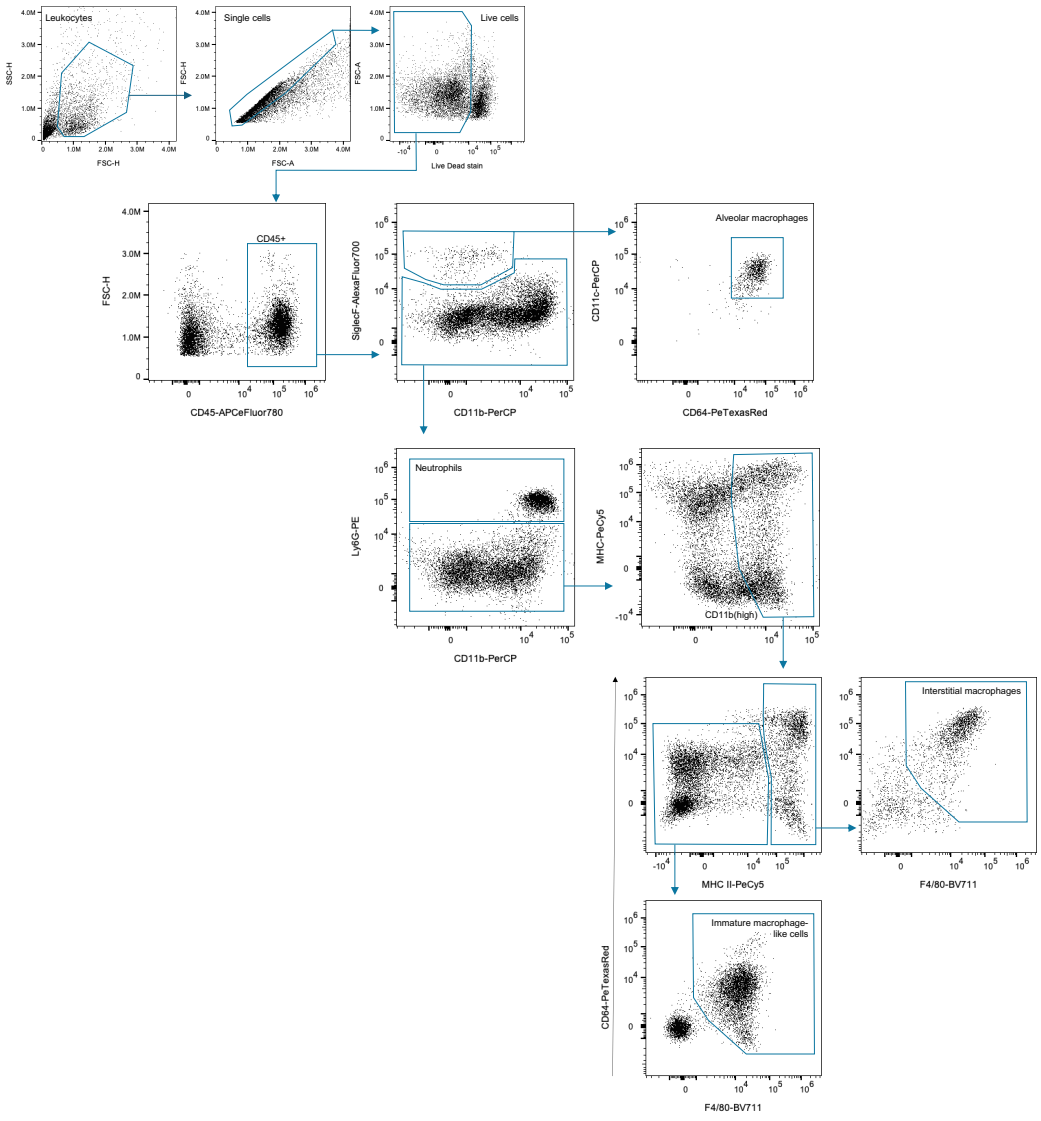
Supplementary Figure 3. 3 - Gating strategy of activated CD4 T, regulatory T cells, CD8 T and B cells. Plots for spleen samples. Gating strategy in blood, lung and peritoneal lavage fluid follows the same strategy.



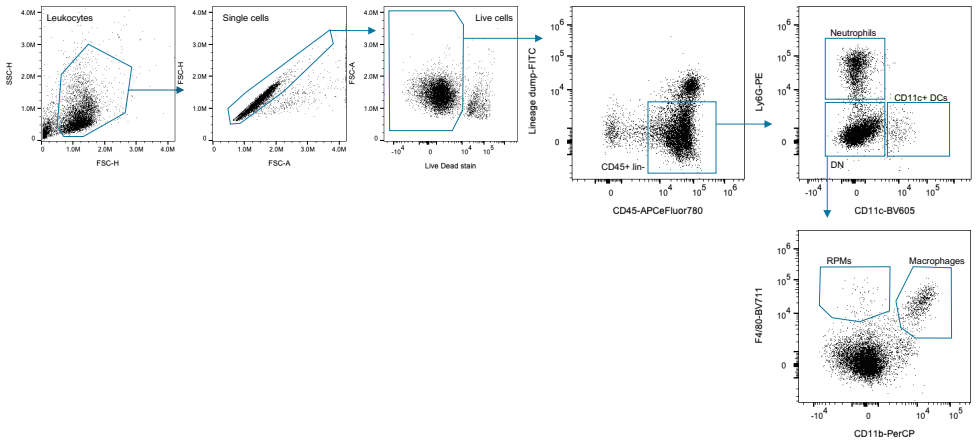
Supplementary Figure 3. 4 - Gating strategy of spleen activated NKT and NK cells. Plots for spleen samples. Gating strategy in blood, lung and peritoneal lavage fluid follows the same strategy.



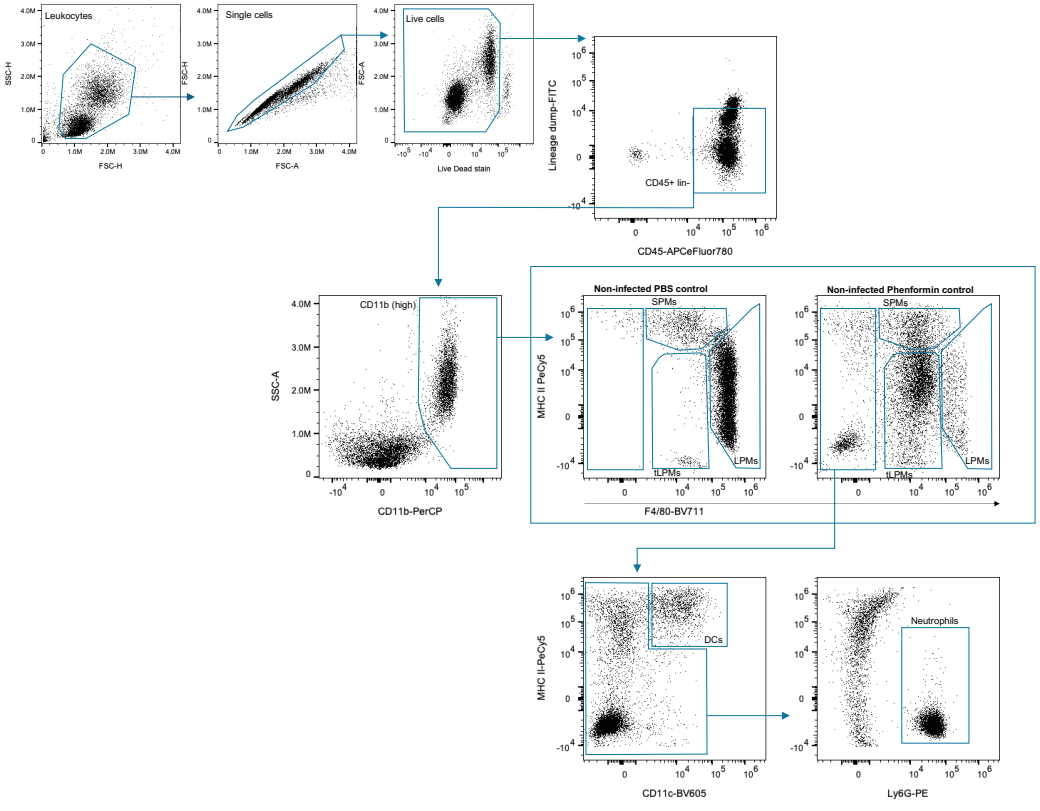
Supplementary Figure 3. 5 - Gating strategy of blood neutrophils, CD11c+ dendritic cells, eosinophils, Ly6C monocytes and macrophages.



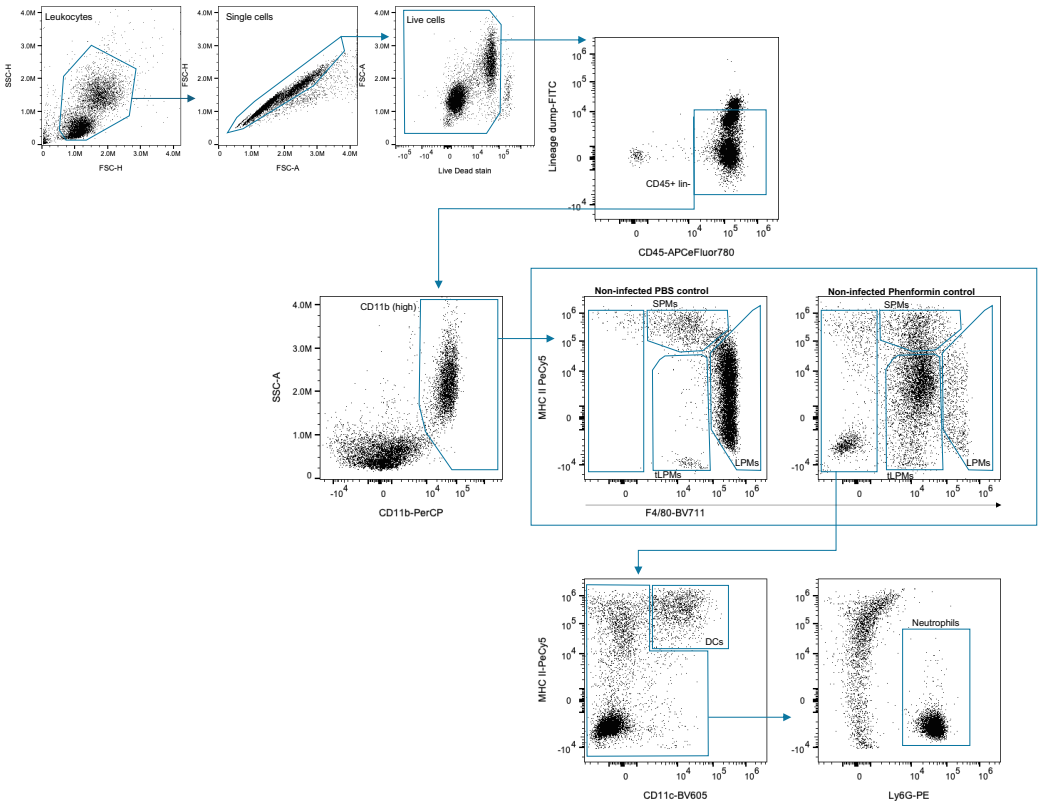
Supplementary Figure 3. 6 - Gating strategy of lung alveolar macrophages, neutrophils, immature macrophage-like cells and interstitial macrophages.



Supplementary Figure 3. 7 - Gating strategy of spleen neutrophils, CD11c+ dendritic cells, red pulp macrophages (RPM) and macrophages.



Supplementary Figure 3. 8 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, transitioning large peritoneal macrophages and large peritoneal macrophages.



Supplementary Figure 3. 9 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, transitioning large peritoneal macrophages and large peritoneal macrophages.

Chapter 4

Contribution of phenformin-induced immune cell population changes to tolerance and resistance mechanisms

Author contributions

Kátia Jesus was responsible for planning, executing and running analysis scripts on data from all experiments in this chapter. Miguel Mesquita provided assistance in the *in vivo* work, panel design, and flow cytometry data analysis. André Barros developed the scripts for statistical analysis. Luís Ferreira Moita supervised the project and reviewed this chapter.

4.1. Abstract

Sepsis is characterized by an extensive dysregulation of the host immune response, consisting of a hyperinflammatory early stage followed by immune paralysis. This ultimately compromises the response to infection but is also a major cause of extensive tissue damage and lack of repair programs. In the previous chapters, we described our observations that phenformin, which inhibits complex I of the ETC and induces mild mitochondrial stress, protects mice from sepsis. This protection is firstly associated with lower tissue damage and lower lymphocyte and NK cell population activation, followed by a shift in the immune program towards more efficient pathogen clearance, which seems to correlate with faster recruitment of neutrophils and monocytes.

In an attempt to understand the pathophysiological role of lymphocytes in sepsis and the dependency of control of tissue damage by phenformin with these cell populations, we performed adoptive cell transfers in wild-type animals. We used a mouse model that lacks lymphocyte populations (B6.Rag2 KO). To understand if innate immune cell recruitment is dependent on a modulation of the hematopoietic process, we immunophenotyped bone marrow progenitors and peritoneal cavity cells after phenformin administration.

Our data shows that lymphocytes have a limited role in the observed tissue damage in our sepsis model. Therefore, we hypothesize that NK cells may play a major role in tissue damage associated with sepsis pathophysiology. We have not tested this hypothesis in the current work. However, our results show phenformin can modulate the hematopoietic process, promoting emergency myelopoiesis. Future studies characterizing the hematopoietic process after phenformin administration during sepsis could help elucidate whether this is a protective mechanism associated with trained innate immunity.

4.2. Introduction

Sepsis can be defined as a maladaptive immune response of the host to an infection[1]. It is characterized by a first pro-inflammatory burst that results in extensive tissue damage, followed by immune paralysis due to cell exhaustion, which impairs tissue repair mechanisms. Treatment strategies are still not specific and efficient enough to tackle these major concerns[2].

While innate immune cells are the first responders in infection and usually display a more aggressive phenotype, the orchestration and maintenance of inflammation in conditions of extensive tissue damage is often the result of lymphocyte-derived signals[3]. In fact, CD4 and

CD8 T cells play crucial roles in the pathophysiology of asthma, allergic reactions, obesity, and autoimmune conditions[4–6]. Furthermore, studies also indicate a potential in mitochondrial control of exacerbated inflammation in these populations[7]. Adding to this, NK cells, which are innate lymphoid cells, are perceived as more aggressive and quicker in their response to an insult and extensively exert control in initiating, maintaining, and regulating inflammation[8]. However, research on the role of mitochondria in the inflammation exacerbation by NK cells is scarce and incomplete[9,10].

The immune paralysis stage of sepsis is driven by impaired capacity to mount an appropriate immune response to the challenge, ultimately resulting in increased susceptibility to secondary or ongoing unresolved infection. This can be associated with a dysregulation of hematopoiesis[11].

Hematopoiesis in the bone marrow is fundamental for keeping organismal homeostasis. When an insult occurs, hematopoiesis is a key biological process for orchestrating proper immune responses, promptly accelerating the differentiation of immune cells required to resolve the challenges[12]. Myelopoiesis is the process by which myeloid lineage cells are differentiated and is comprised of a series of steps, during which hematopoietic stem cells (HSCs) self-renew and produce multipotent progenitors that support the daily upkeep of blood immune cells. Although maintaining the capacity to differentiate into multiple subsets, multipotent progenitors (MPPs) can further be divided into subclasses biased towards specific mature cell types. Multipotent progenitors 3 (MPP3) are biased towards myeloid cell lineage differentiation. During infection or stress, emergency myelopoiesis mechanisms prioritize myeloid lineage differentiation to compensate for the conditions' demand of these cells[12]. A growing body of evidence shows modulation of myeloid progenitors could have a fundamental role in trained innate immunity and the resolution of systemic inflammation[13–15].

In this chapter, we aimed to understand the physiological impact of the reduction of expression of the early activation marker CD69 on lymphocyte populations by phenformin during sepsis. We further investigated whether phenformin-induced recruitment of monocytes to the peritoneal cavity following macrophage disturbance of homeostasis reaction resulted from a modulatory effect of our treatment in the hematopoietic process.

Our data suggests that the exacerbated tissue damage observed in our sepsis model, and the protection that phenformin confers are not a result of CD8 T effector function as no differences in protective phenotype of phenformin through disease tolerance were observed with adoptive cell transfer of CD8 T cells or in B6.Rag2 KO mice. Further studies should focus on understanding whether NK cells are the main drivers of tissue damage. On the other hand,

we have found compelling evidence for a phenformin-driven modulation of the hematopoietic process towards an emergency myelopoiesis. This results in rapid recruitment of neutrophils and monocytes to the peritoneal cavity after phenformin injection. This encourages more detailed studies to further understand the significance of this pre-emptive recruitment of innate immune cells to the site of infection initiation and in possible resistance mechanisms.

4.3. Methods

4.3.1. Experimental Models

4.3.1.1. Mice

Animal studies were performed following Portuguese regulations and approved by the ethics committee of Instituto Gulbenkian de Ciência, ORBEA, and national regulatory agency, DGAV, under the project reference number A011.2019. All experiments were performed in the Mouse Facility of the Instituto Gulbenkian de Ciência.

C57BL/6J and B6.Rag2 KO male mice were obtained from the production unit of the animal facility. All animals used were 8- to 12-weeks-old unless otherwise stated. In all experiments, age-matched animals were randomly assigned to distinct experimental groups.

Animals were housed in specific pathogen-free (SPF) conditions, with 12 hours light/dark cycles, 50-60 % humidity, and 21-23 ºC temperature. Food and water were available *ad libitum* in all experiments.

4.3.1.2. Primary cell culture of bone marrow-derived macrophages (BMDMs)

Bone marrow-derived macrophages were differentiated from cells of 8- to 12-week-old C57BL/6J mice. For this, mice were euthanized by CO_2 inhalation, disinfected with 70 % ethanol, and the hind limbs were collected for tibia and femur recovery. After careful removal of muscle, the extremities were cut, and bone marrow cells were flushed out with 1 mL RPMI1640 using a 30 G needle insulin syringe. Collected cells were pelleted by centrifugation at 320 x g for 5 min. Supernatant was discarded and cells were resuspended in 10 mL RPMI1640 supplemented with 10 % (v/v) FBS, 1 % (v/v) Penicillin/Streptomycin, 0.1 % (v/v) β-mercaptoethanol, 30 % (v/v) L929 supernatant. After counting with trypan blue to assess viability, cells were resuspended in the appropriate volume of complete media to obtain a cell

concentration of 4 x 10^5 cells/mL. Cell suspensions were plated in Petri dishes at a final density of 4 x 10^6 and incubated for 6 days at 37 ºC and 5 % CO_2 for differentiation. On the 6th day, cells were assessed visually for contaminations and differentiation success. Media was replaced with fresh non-supplemented RPMI1640, and cells were detached using a cell scraper. Cell homogenate was centrifuged at 320 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in 10 mL RPMI1640 supplemented with 10 % (v/v) FBS, 1 % (v/v) Penicillin/Streptomycin, 0.1 % (v/v) β-mercaptoethanol, 5 % (v/v) L929 supernatant for cell counting with trypan blue to assess viability. Cells were replated at a density of 5 x 10^5 cells/well in 12-well plates and incubated overnight at 37 ºC and 5 % CO_2 to adhere for *in vitro* clearance assays. On the following day, supernatant media was removed, cells were washed with 1 x PBS and fresh RPMI1640 supplemented with 10 % (v/v) FBS, 1 mM Pyruvate, 2 mM Glutamine, 0.1 % (v/v) β-mercaptoethanol was added.

4.3.1.3. Bacterial cultures

See section 2.3.1.2..

For *in vitro* experiments, *E. coli* CFP-tagged with resistance to streptomycin was used.

4.3.2. Methods details

4.3.2.1. Bacterial peritonitis model of mild sepsis using *E. coli* and drug treatments

See section 2.3.2.1. .

4.3.2.2. Colony forming units (CFUs) assay

See section 2.3.2.2. .

4.3.2.3. Biochemical assays of mouse serum

Circulating damage markers were quantified by an external service provider, DNATech. Serum samples were collected and sent on the day of collection on ice. Parameters determined were

Aspartate Transaminase (AST), Alanine Transaminase (ALT) and Lactate Dehydrogenase (LDH).

4.3.2.4. Adoptive cell transfer experiments

To understand the role of spleen cells in the tissue damage observed in our disease models, we performed adoptive cell transfer experiments. Here, either whole spleen cell suspensions or specific cell populations isolated from spleens of donor animals infected and treated with phenformin or vehicle 24 h post-infection and injections were collected and processed as described in section 3.3.2.2., under sterile conditions for retro-orbital injection of approximately 1 - 2 x 10^6 cells into age-matched recipient naïve animals. Cell suspension for injection was resuspended in sterile 1 x PBS supplemented with 2 % (v/v) FBS. Donor and recipient animal serum samples were collected 24 h post injections and circulating damage markers were assessed as described in section 4.3.2.3.

4.3.2.5. CD8 T cell negative magnetic isolation

CD8 T cells were isolated by negative magnetic selection from spleen cells using the MojoSort™ Mouse CD8 T cell Isolation Kit (BioLegend Cat#480006) with LS MACS columns and the MACS multi-stand magnet, following the manufacturer's recommendations for the column protocol.

Spleens were ground into single-cell suspension by using tweezers and sterilized mesh to disrupt the capsid and dissociate the cells in RPMI1640. Cells were pelleted by centrifugation at 320 x g for 5 min and resuspended to a concentration of 1 x 10^8 cells/mL in 1 x PBS 2 % (v/v) FBS (from here on referred to as selection buffer).

Cell suspensions were incubated with the pre-diluted Biotin-Antibody Cocktail at the recommended concentration (10 μL Biotin-Antibody cocktail for 10^7 total spleen cells) for 15 min on ice. Cells were washed with selection buffer and resuspended to 100 μL buffer per 10^7 cells and incubated with the recommended concentration of pre-diluted Streptavidin Nanobeads (10 μL streptavidin nanobeads for 10^7 total spleen cells) for 15 min on ice. After incubation cells were washed with selection buffer, and centrifuged at 320 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in at least 500 μL selection buffer. LS MACS columns were placed on the MACS multi-stand magnet and rinsed with 3 mL selection buffer. Cell suspensions were run through the separation column, and flow-through of untouched cells (CD8 T cell fraction) was collected. Columns were washed with 3 mL

selection buffer and flow-through was collected and added together with the untouched cells fraction. When needed, labeled cells were also collected by removing the column from the magnetic separator, adding separation buffer, and flushing out with the plunger. Cells were resuspended and plated at the appropriate cell density in RPMI1640. Cell isolation purities were assessed by flow cytometry by staining for cell population-specific markers.

4.3.2.6. Flow cytometry sample preparation

Immunophenotyping of cell populations was performed in spleen, peritoneal cavity, and bone marrow cells. For spleen samples, please see section 3.3.2.2.. Peritoneal and bone marrow, samples were collected at the indicated time points (8 and 24 h post-treatment) and processed as described.

For peritoneal cavity cell collection, mice were disinfected with 70 % ethanol after euthanasia by CO_2 inhalation, and an incision was made in the outer skin to expose the inner skin of the peritoneum without rupturing. For cell recovery, 5 mL of FACS buffer were injected into the peritoneal cavity with a 27 G needle. Peritoneal cells were dislodged by massaging the area. Peritoneal fluid was recovered with a 19 G needle. When required, a red blood cell lysis step followed peritoneal cavity cell recovery. Otherwise, samples were centrifuged and resuspended in 1 mL FACS buffer for staining.

For bone marrow cell collection, mice were euthanized by CO_2 inhalation, disinfected with 70 % ethanol and the hind limbs were collected for tibia and femur recovery. After careful removal of muscle, the extremities were cut, and the bones were placed in a 0.5 mL collection tube previously perforated at the bottom with a 19 G needle and placed over a 1.5 mL reaction tube. Samples were centrifuged at 800 x g, 4 ºC for 10 seconds. After centrifugation, cells were resuspended in RBC buffer, briefly incubated, then FACS buffer was added, and cells were centrifuged at 320 x g, 4 ºC for 5 min. Supernatant was discarded, and the pellet was resuspended in 1 mL of FACS buffer for staining.

Unless otherwise specified in the protocol, all samples collected for flow cytometry staining were kept on ice from the moment of dissection until acquisition on a cytometer.

4.3.2.7. Flow cytometry staining

For immunophenotyping of immune cell populations, spleen samples followed the protocol described in section 3.3.2.3.. Peritoneal cavity and bone marrow cells were plated into 96 well

plates at an adequate concentration. Cell suspensions were centrifuged at 320 x g, 4 ºC for 5 min, supernatant was discarded, and peritoneal cavity pellets were resuspended in TruStain Fc block reagent, diluted in 1 x PBS. Bone marrow samples were incubated with the CD16/32 labeled antibody for 25 min on ice. Cells were then washed with 1 x PBS, centrifuged supernatant was discarded, and pellets were resuspended in the appropriate antibody mixes diluted in 1 x PBS and incubated for 25 min on ice in the dark. Antibodies used are listed in the table below. When biotinylated antibodies were used to stain cells, an extra incubation with labeled streptavidin for 25 min on ice. Peritoneal cavity and bone marrow samples were acquired without fixation immediately after collection and staining on a Cytek Aurora spectral cytometer (equipped with four lasers (405nm, 488 nm, 561 nm, 640 nm), two SSC detectors, and 48 fluorescence detectors).

Unmixing was performed with autofluorescence extractions using the unstained control of the specific tissue with the highest autofluorescence profile and zombie Live-Dead controls from the respective tissue. Results were analyzed using FlowJo software (version 10.10.0).

Table 2- Antibodies and reagents used for flow cytometry stainings.

Epitope	**Clone**	**Fluorophore**	**Manufacturer**	**Catalogue Number**
CD4	GK1.5	PE/Cyanine7	BioLegend	100422
CD8a	53-6.7	PE/Cyanine5	BioLegend	100710
CD19	6D5	Brilliant Violet 711	BioLegend	115555
CD69	H12F3	PE	BioLegend	104508
CD25	PC-61	AlexaFluor647	IGC	
FoxP3	FJK-16s	eFluor450	Invitrogen	48-5773-82
Sca1	E13-161.7	FITC	BioLegend	122505
Ly6C	HK1.4	PerCPCy5.5	BioLegend	1280012
CD135	A2F10	PerCPeF710	eBioscience	46135180
CD115	AFS98	PE	BioLegend	135505
TIM4	REA999	PeVio615	Miltenyi Biotech	130-116-638
CD34	MEC14.7	PE/Cyanine5	BioLegend	119311

Streptavidin		PE-Fire700	BioLegened	405174
CD3	17A2	Biotin	BioLegend	100243
CD19	1D3	Biotin	BD Pharmingen	553784
CD48	HM48-1	PeVio770	Miltenyi Biotech	130-102-363
cKit	2B8	APC	BD Pharmingen	553356
F4/80	BM8	AlexaFluor647	BioLegend	123122
Ly6G	1A8	AlexaFluor700	Invitrogen	56-9668-82
CD45	30-F11	APCeFluor780	Invitrogen	47045182
CD16/32	93	BV421	BioLegend	101331
CD150	mShad150	eFluor450	Invitrogen	48-1502-82
MHC II	M5/114.15.2	eFluor506	eBioscience	69532182
Zombie aqua Fixable viability kit			BioLegend	423102
Live/Dead fixable yellow dead cell stain kit			Invitrogen	L34949
CD11c	N418	Brilliant Violet 605	BioLegend	117334
CD11b	M1/70	PerCP	BioLegend	101229
CD11b	M1/70	BV785	BioLegend	101243
TruStain (Fc block)		Unconjugated	BioLegend	101320

4.3.2.8. BMDM infection clearance assay

To understand if the presence of phenformin influences the clearance capacity of macrophages, cells were prepared as described in section 4.3.2.1. For this, 5 x 10^5 cells were plated in 12-well plates. On the day of the experiment, when required, the media of the cells was replaced with fresh RPMI1640 supplemented with 10% (v/v) FBS. For infection, 5 - 10 x

10^5 *E. coli*-CFP grown as described in section 2.3.1.2., were added to the cells in the presence or absence of 100 μg/mL phenformin. Cells were collected at 0, 1, 2, and 4 h post-infection and treatment. For macrophages, cells were scraped off before collection. Cell suspensions were collected and centrifuged at 320 x g, 4 ºC for 5 min, and the pellet was resuspended in 1 x PBS for FACS staining. Cells were stained following the protocol described in section 3.3.2.3., until fixation. Samples were acquired without fixation immediately after collection and staining on a Cytek Aurora spectral cytometer (equipped with four lasers (405nm, 488 nm, 561 nm, 640 nm), two SSC detectors, and 48 fluorescence detectors). Unmixing was performed with autofluorescence extractions using the unstained control with the highest autofluorescence profile. Clearance capacity was analyzed by assessing the expression of CFP on target cells.

4.3.2.9. Data modeling and statistical analysis

All data was analyzed using R studio software. For visualization, the ggplot2 and ggpubr packages were used.

For survival analysis, Cox proportional hazard linear regression models, with random effect structure (1|Experiment), was adopted using the Coxme package. When the experimental layout required sacrificing groups of animals at specific time points, data was analyzed with censored events (including only groups of animals that were not sacrificed because of experimental read-outs). This criterion was also employed for visualization in representative plots.

For vital parameters analysis, including temperature, weight, and glucose, linear regression, with random effect structure (1|Experiment), one per time point was performed. This prevents the model from estimating parameters for animals that have succumbed to disease and thus cease to exist at later time points.

For pathogen loads, CFUs were analyzed by modeling the effect of treatment across the different time points for each independent experiment through mixed-effect zero-inflated negative binomial regression.

Circulating damage markers were analyzed using linear regression and pairwise comparisons.

For flow cytometry, results in percentages were analyzed by linear regression in logit-transformed data, whilst absolute count results were analyzed by negative beta-binomial

model to determine the effect of treatment across the different time points independent of experiment.

4.4. Results

4.4.1. Phenformin does not appear to skew T helper cell differentiation fate during infection

In Chapter 3, we described that phenformin can downregulate the expression of CD69 during infection in various subsets of lymphocytes and NK cells. Considering these results and our speculation that the downregulation of activation marker CD69 might be associated with lower tissue damage observed in treated mice, thus involved in mechanisms of disease tolerance, we have decided to assess the possible impacts of the downregulation of this marker in the T cell subset differentiation modulation.

Within T helper cell populations, an increasing number of studies show that mitochondrial homeostasis could play essential roles in cell differentiation, proliferation, and maturation[16–18]. In fact, there is growing interest in understanding how inhibition or promotion of specific electron transport chain complexes could shape T cell populations and thus be used for protection in complex conditions driven by erroneous T cell responses, such as autoimmune disease or even cancer[17,19]. Inhibition of mitochondrial ATP synthase in particular, has been associated with fate decision between pathogenic Th17 and regulatory T cells[20]. In line with this, a study has shown that co-administration of tacrolimus with metformin could improve immune homeostasis after solid organ transplant by promoting an increase of T regulatory cells and reduction of Th17 cells[21]. Thus, we investigated whether, during infection, complex I ETC inhibition by phenformin was skewing CD4 T cell differentiation towards a regulatory profile by assessing the levels of Foxp3 regulatory cells in the spleen.

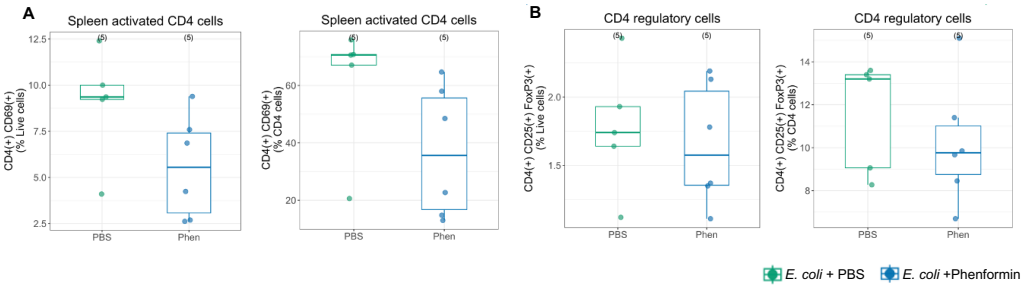


Figure 4. 1 - Phenformin does not significantly impact FoxP3 regulatory cells in the spleen.

Quantification of percentages, from left to right of, spleen **(A)** activated CD4 within live cells (left side), and within CD4 parent population (right side) and **(B)** regulatory FoxP3 CD4 cells within live cells (left side), and within CD4 parent population (right side)**. (A, B)** represent pooled data of two experiments, individual mice measured are represented by points and box plots indicate quartiles and median. Gating strategy can be found in Supplementary Figure 3.3. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Figure 4.1 shows that phenformin does not appear to significantly impact the percentage of regulatory cells within the CD4 T cell population at 24 h post-infection and treatment.

4.4.2. Damage marker profiles cannot be replicated by adoptive cell transfers from infected donor mice to naïve recipient mice

Considering the results shown in Chapter 3 and the extensive literature supporting the role of overactivation of lymphocytes in exacerbated tissue damage, we hypothesize that phenformin-treated mice have lower tissue damage due to the lower activation profiles of their lymphocyte populations, as observed by decreased expression of CD69.

To test this, we performed whole splenocyte cell transfer experiments from infected into naïve mice. For this, spleens of C57BL/6J mice infected and treated with vehicle solution or phenformin, as described in section 2.3.2.1., were collected under sterile conditions 24 h after infection. Spleens were processed as described in section 4.3.2.4. and injected retro-orbitally into naïve mice. After 24 h of cell transfer naïve mice were sacrificed. Blood samples were collected from donors and recipients and serum was harvested for circulating damage marker quantification. Results are shown in Figure 4.2.

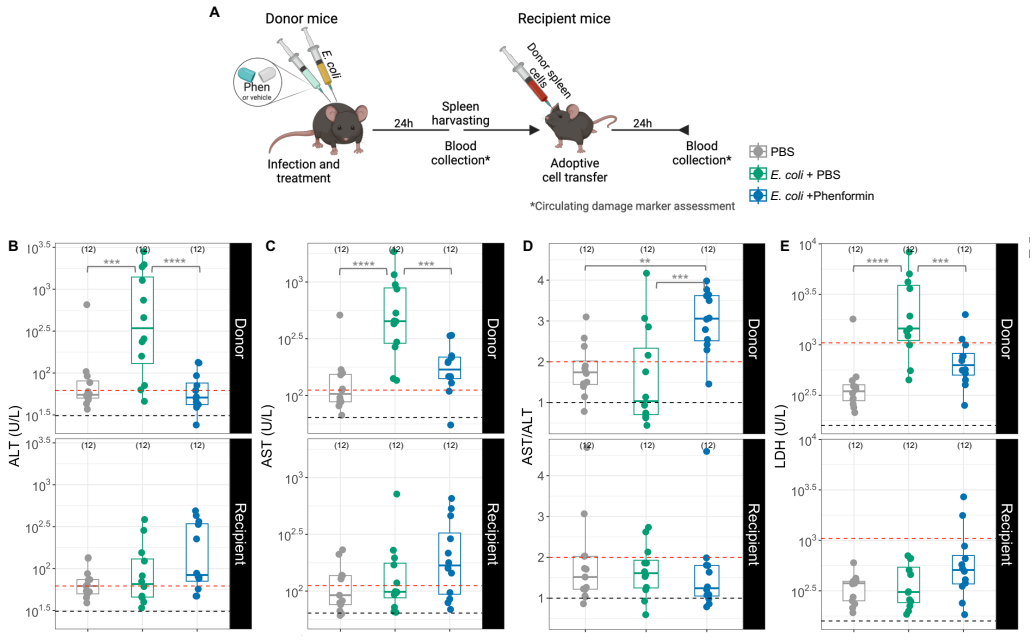


Figure 4. 2 - Adoptive cell transfers of whole spleen cells do not replicate damage marker kinetics in naïve mice.

(A) Schematic representation of the experimental layout. **(B-D)** Concentrations of the organ damage markers alanine transaminase (ALT), aspartate transaminase (AST), ratio of AST/ALT, lactate dehydrogenase (LDH), in circulating blood of male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) (Donors) or 24h after whole splenocyte cell transfer (recipients). Plots represent pooled data from three independent experiments, individual mice measured are represented by points and box plots indicate quartiles and median. Red and black dashed lines indicate maximum and minimum reference values, respectively. **Statistical analysis** was done using R, by linear regression, and pairwise comparison. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Data indicates that infection highly increases circulating damage markers such as alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) when compared to control non-infected vehicle-injected mice. Furthermore, phenformin treatment greatly reduced the levels of these circulating damage markers, as observable in Figure 4.2 (A-D), upper plots. However, cell transfers into recipient naïve mice did not yield comparable levels of circulating damage markers, and no significant difference in the levels of the markers is visible between the recipients of donors from the different groups, as shown in the lower plots.

The spleen is composed of a highly heterogeneous collective of immune cells, and previous results in this study indicate that phenformin impacts cell types in different fashions. Thus, to reduce the noise ratio of the varying cell populations, we have proceeded by using isolated CD8 T cells from the donor mice spleens for adoptive cell transfer. Cytotoxic T cells were the

population of lymphocytes showing greater reduction of CD69 levels at earlier time points. This strategy would allow us to address the impact of CD69 reduction on CD8 effector function in a more refined manner.

In line with what was observed in whole splenocyte cell transfer experiments, results in Figure 4.3 (A-D) show that even though donors of CD8 T isolated cells that were treated with phenformin have lower circulating damage markers when compared to vehicle-treated infected mice, this profile is not transferable to the recipients of these cells. Of note, although the difference between treated and non-treated donor animals is still observable, in these experiments, phenformin-treated mice had a greater variability in the levels of circulating damage marker, and differences are not as significant as in previous experiments.

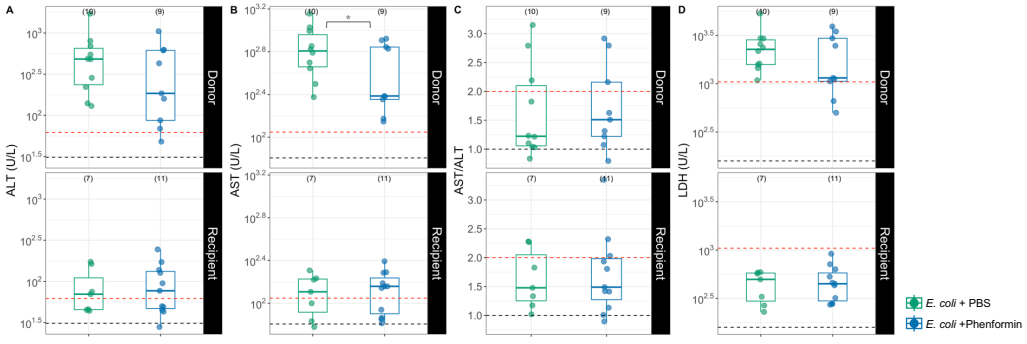


Figure 4. 3 - Adoptive cell transfer of CD8 T lymphocytes does not replicate circulating damage marker kinetics in naïve mice.

(A-D) Concentrations of the organ damage markers alanine transaminase (ALT), aspartate transaminase (AST), ratio of AST/ALT, lactate dehydrogenase (LDH), in circulating blood of male C57BL/6J mice with 3- 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) (Donors) or 24 h after splenocyte CD8 cell transfer (recipients). **(A to D)** represent pooled data of two independent experiments, individual mice measured are represented by points and box plots indicate quartiles and median. Red and black dashed lines indicate maximum and minimum reference values, respectively. **Statistical analysis** was done using R, by linear regression and pairwise comparison. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

Altogether, the low levels of tissue damage induced by the transferred cells do not allow us to causally demonstrate that phenformin-treated septic mice have lower tissue damage scores due to their lower lymphocyte activation. These low levels might be a constraint of the experimental design due to the short time frame from the moment the cells are transferred into the naïve mice to damage quantification. Another important issue could be related to the use of naïve mice for recipients. Activated T cells, at least at this early stage, might need other environmental factors to keep their exacerbated response, and the phenotype characteristic of sepsis might, in fact, be the result of a systemic response rather than an isolated population.

4.4.3. Depletion of T lymphocyte population does not decrease sepsis-associated tissue damage or phenformin protection

To understand whether lymphocytes are involved in the tissue damage of sepsis and if the disease tolerance mechanisms triggered by phenformin rely on lower lymphocyte activation, we used an animal model that does not have these immune cell populations, the B6.Rag2 KO. This will allow us to understand (1) if phenformins' disease tolerance triggering capacity is dependent on lymphocyte populations; (2) if the resistance mechanisms are maintained without lymphocytes.

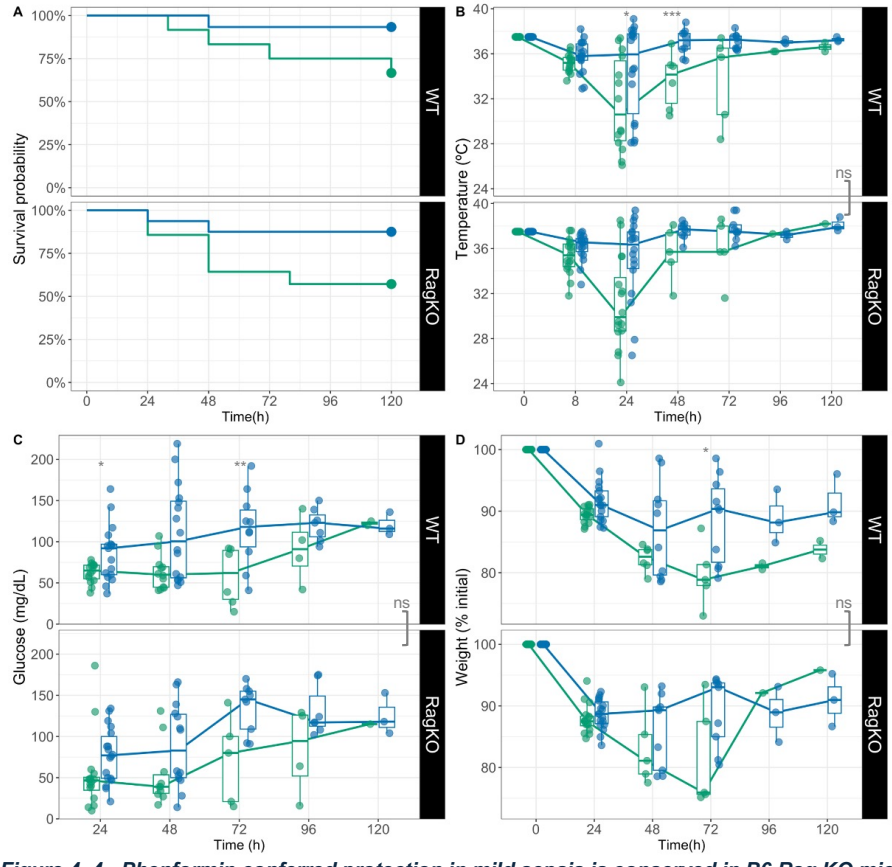


Figure 4. 4 - Phenformin conferred protection in mild sepsis is conserved in B6.Rag KO mice.

Survival **(A)**, rectal temperature **(B),** glucose **(C)**, and % initial body weight **(D)** after infection of male C57BL/6J (upper plots) and B6.Rag2KO (lower plots) mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) at 0, 24, 48, 72, 96 and 120 h. (A) represents pooled data from 2 independent experiments. (B-D) represent mean ±SD pooled from 2 independent experiments, individual mice measured are represented by points and box plots indicate quartiles and median. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time-points independent of experiment.

Temperature, glucose, and weight were analyzed by linear regression. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****), not-significant (ns).

Results from Figures 4.4 to 4.6 show that even in the absence of lymphocyte populations, phenformin confers protection against the infection of septic mice. B6.Rag2 KO mice show improved survival and faster vital parameter recovery from infection, and the kinetics of recovery are very similar to those of control wild-type mice (Figure 4.4 (A-D)). Surprisingly, B6.Rag2 KO mice also show similar increases in circulating damage markers upon infection, indicating that other cells might be inducing tissue damage. In line with this, results show that phenformin treatment significantly reduces damage marker levels (Figure 4.5 (A-D)). Together these data indicate that lymphocytes are not the major drivers of tissue damage in sepsis at the time frame being considered. Furthermore, pathogen clearance kinetics are similar in B6.Rag2.KO mice and wild type (Figure 4.6). Confirming that pathogen clearance is also independent of lymphocyte populations.

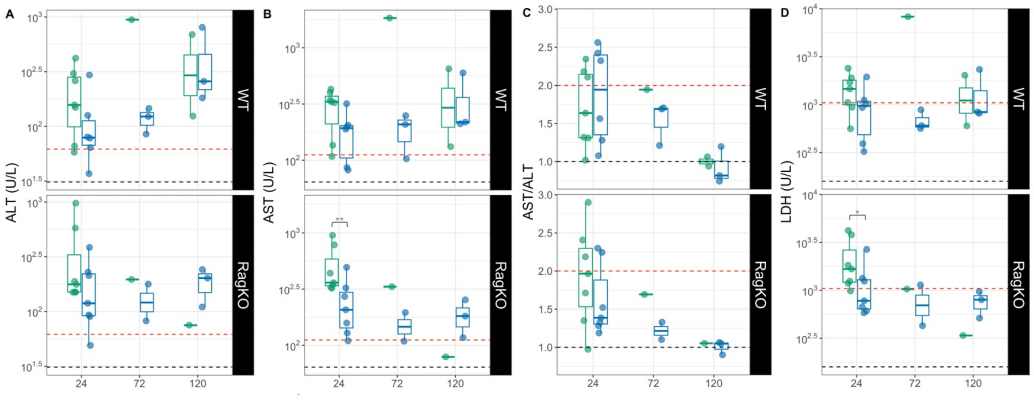


Figure 4. 5 – B6.Rag2 KO mice show similar levels of damage to wild-type animals.

(A-D) Concentrations of the organ damage markers alanine transaminase (ALT), aspartate transaminase (AST), ratio of AST/ALT, lactate dehydrogenase (LDH), in circulating blood of male C57BL/6J (upper plots) and B6.Rag2KO (lower plots) mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control). **(A-D)** represent pooled data of two independent experiments, individual mice measured are represented by points and box plots indicate quartiles and median. **Statistical analysis** was done using R, by linear regression and pairwise comparison. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).

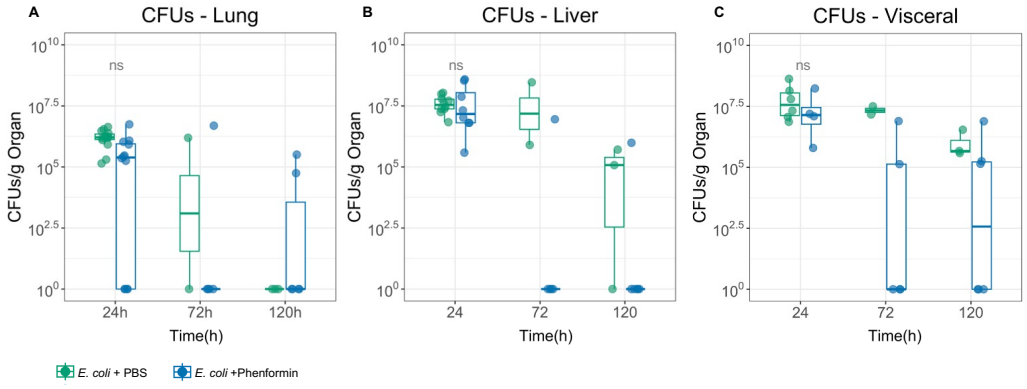


Figure 4. 6 – Bacterial clearing in the absence of lymphocytes follows similar kinetics

Bacterial load in mouse B6.Rag2KO lung **(A)**, liver **(B)**, visceral adipose tissue **(C),** at the indicated time points after infection. **(A-C)** circles represent individual mice from at least two independent experiments, and box plots indicate minimum, maximum, and mean. **(A-C)** represent pooled data from at least two independent experiments; individual mice measured are represented by points, and box plots indicate quartiles and median. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time points for each independent experiment, individual mice measured are represented by points and box plots indicate quartiles and median. CFUs were analyzed by mixed-effect zero-inflated negative binomial regression. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****) in which black * indicate differences in CFU counts and teal blue * indicate differences in zero CFUs, not-significant (ns).

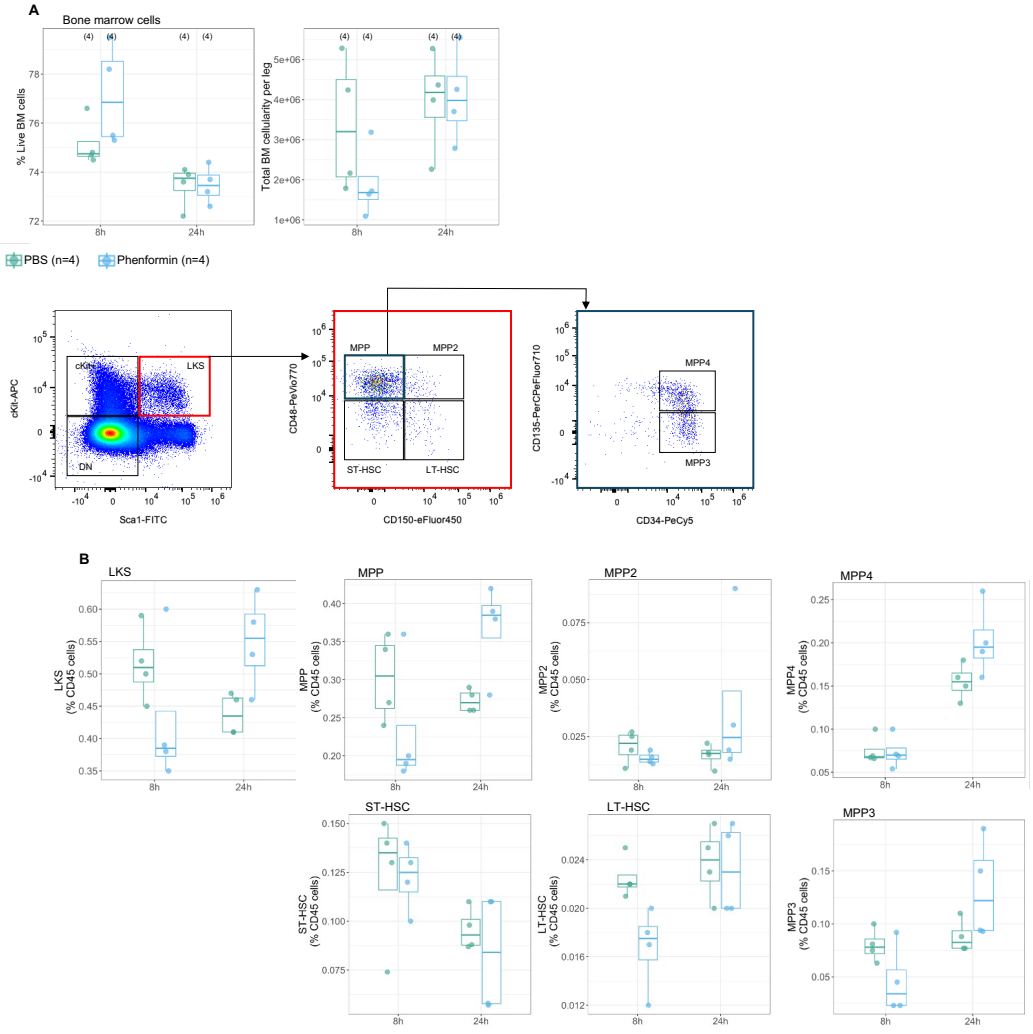
4.4.4. Phenformin-induced myelopoiesis as a booster for the immune system against infection

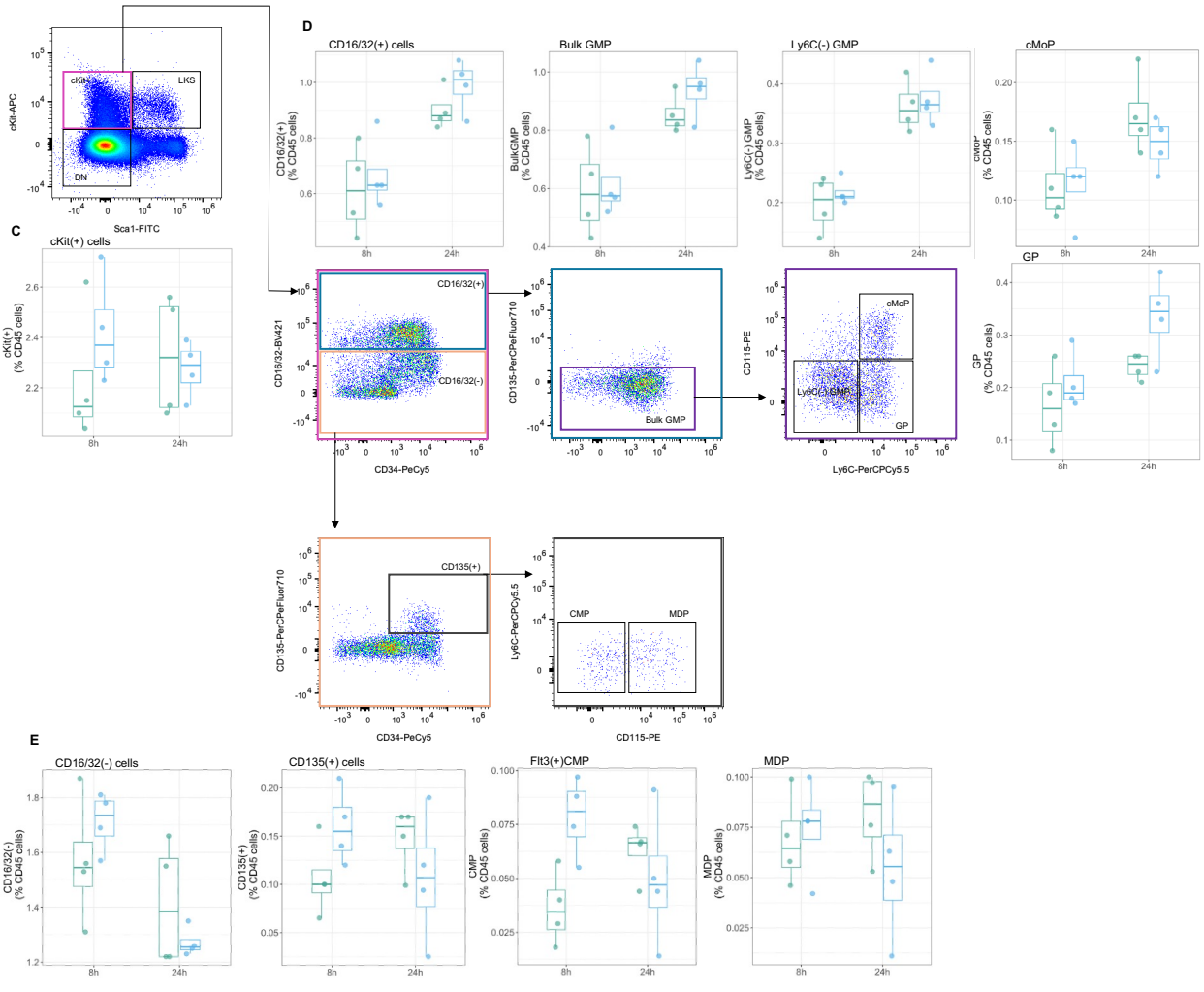
As described in Chapter 3, our data suggested an overall change in viable cell percentages and total cellularity across tissues, as well as a modulation of the immune cell profiles. This is most remarkably visible in the peritoneal cavity, with differences in myeloid-derived immune cell populations even without infection stimulation. More specifically, our results indicate that phenformin could be triggering a Macrophage Disturbance of Homeostasis Reaction (MDHR). We speculated that this could ultimately be protective in an infection context because it could be a pre-emptive promotion of myelopoiesis. To elucidate whether this phenotype arises from phenformin modulating the hematopoietic process, we injected male C57BL/6J mice with 100 μg/g phenformin or vehicle solution and characterized the progenitors of various populations in the bone marrow and the respective immune cell population in the peritoneal cavity.

In line with what we have observed previously in other tissues, phenformin-treated animals show increased percentages of viable cells in their bone marrow. However, total cell count is reduced compared to vehicle-injected animals (Figure 4.7 (A)). Because of these differences,

we have characterized hematopoietic stem cells and lineage-specific progenitors in percentages of CD45(+) cells for comparison between groups (Figure 4.7 (B-F)).

Our results show that 8 h after phenformin treatment, long-term (LT)-HSC and MPP3 (a multipotent progenitor biased for myeloid cells) are reduced (Figure 4.7 (B)). In line with this, Flt3(+) common myeloid progenitors (CMPs), monocyte-dendritic cell progenitors (MDPs), common monocyte progenitors (cMoP), and granulocyte progenitors are increased (Figure 4.7 (D-E)). At 24 h after phenformin treatment, we can observe an increase in MPP3, MPP2 (megakaryocytic/erythroid-biased progenitors), and MPP4 (lymphoid-biased progenitors), as well as in bulk GMPs, which results in a noticeable granulocyte progenitor increase.




cKit-APC
Sca1-FITC
cKit
LKS
DN
C
cKit(+) cells
cKit(+)
(% CD45 cells)
8h
24h
D
CD16/32(+) cells
CD16/32(+)
(% CD45 cells)
Bulk GMP
BulkGMP
(% CD45 cells)
Ly6C(-) GMP
Ly6C(-) GMP
(% CD45 cells)
cMoP
cMoP
(% CD45 cells)
GP
GP
(% CD45 cells)
CD16/32(+)
CD16/32(-)
CD16/32-BV421
CD34-PeCy5
CD135-PerCPeFluor710
Bulk GMP
CD115-PE
cMoP
Ly6C(-) GMP
GP
Ly6C-PerCPCy5.5
CD135(+)
CD135-PerCPeFluor710
CD34-PeCy5
Ly6C-PerCPCy5.5
CMP
MDP
CD115-PE
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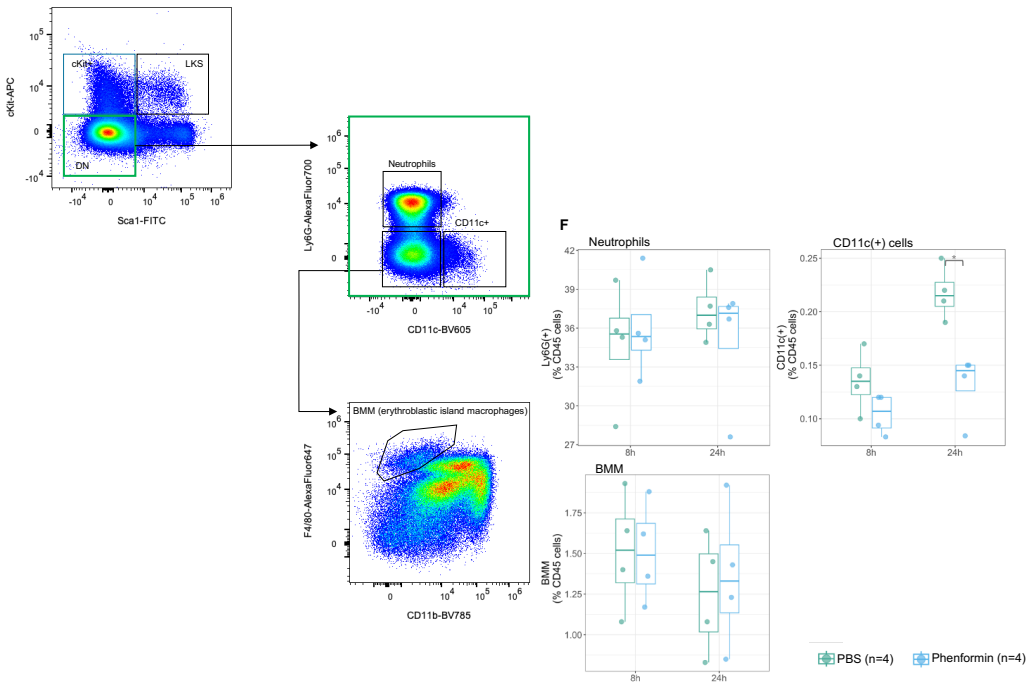

Figure 4. 7 - Phenformin induces emergency myelopoiesis.

(A-F) Myeloid cells and progenitor populations in non-infected male C57BL/6J mice treated with 100 µg/g body weight phenformin (or PBS as a control). Quantification in indicated conditions and time-point of **(A)** live cells (percentage of total events) and live cell count per leg; and of cell population given in percentage of CD45 cells of **(B)** LSK cell subsets, MPP (multipotent progenitors); MPP2 (megakaryocytic/erythroid-biased progenitor); MPP3 (myeloid-biased progenitor); MPP4 (lymphoid-biased progenitor); HSC (Hematopoietic stem cells); LT-HSC (long term - HSC); ST-HSC (short term HSC); **(C))** cKit(+) subsets **(D)** CD16/32(+) subsets, bulkGMP (Granulocyte-monocyte progenitors); Ly6C(-)GMP; cMoP (common monocyte progenitor); GP (granulocyte progenitor); **(E)** CD16/32(-) subsets, CMP (common myeloid progenitors); MDP (monocyte-dendritic cell progenitor) and **(F)** committed neutrophils, BMM (erythroblastic island macrophages) and CD11c cells. **(A-F)** represent pooled data of one experiment, individual mice measured are represented by points and box plots indicate quartiles and median. Gating strategy can be found in Supplementary Figure 4.3. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: $p < 0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), $p<0.0001$(****).

As a result of these changes in lineage progenitors in the bone marrow, we can observe a significant influx of monocytes and neutrophils in the peritoneal cavity (Figure 4.8 (A-D)). This is already observable at 8 h, but, in the case of monocytes, becomes more striking at 24 h post-injection. Small and large peritoneal macrophages disappear from the peritoneal cavity upon phenformin injection, in line with what was described in Chapter 3. Furthermore, by comparing the gating strategy from Chapter 3 with the improved strategy used in Chapter 4 (data not shown), we were able to clarify that the incoming cells in the peritoneum were, in fact, monocytes that are being recruited through phenformin-induced myelopoiesis promotion into the peritoneal cavity. We speculate these cells are maturing towards the peritoneal resident-macrophage phenotype.

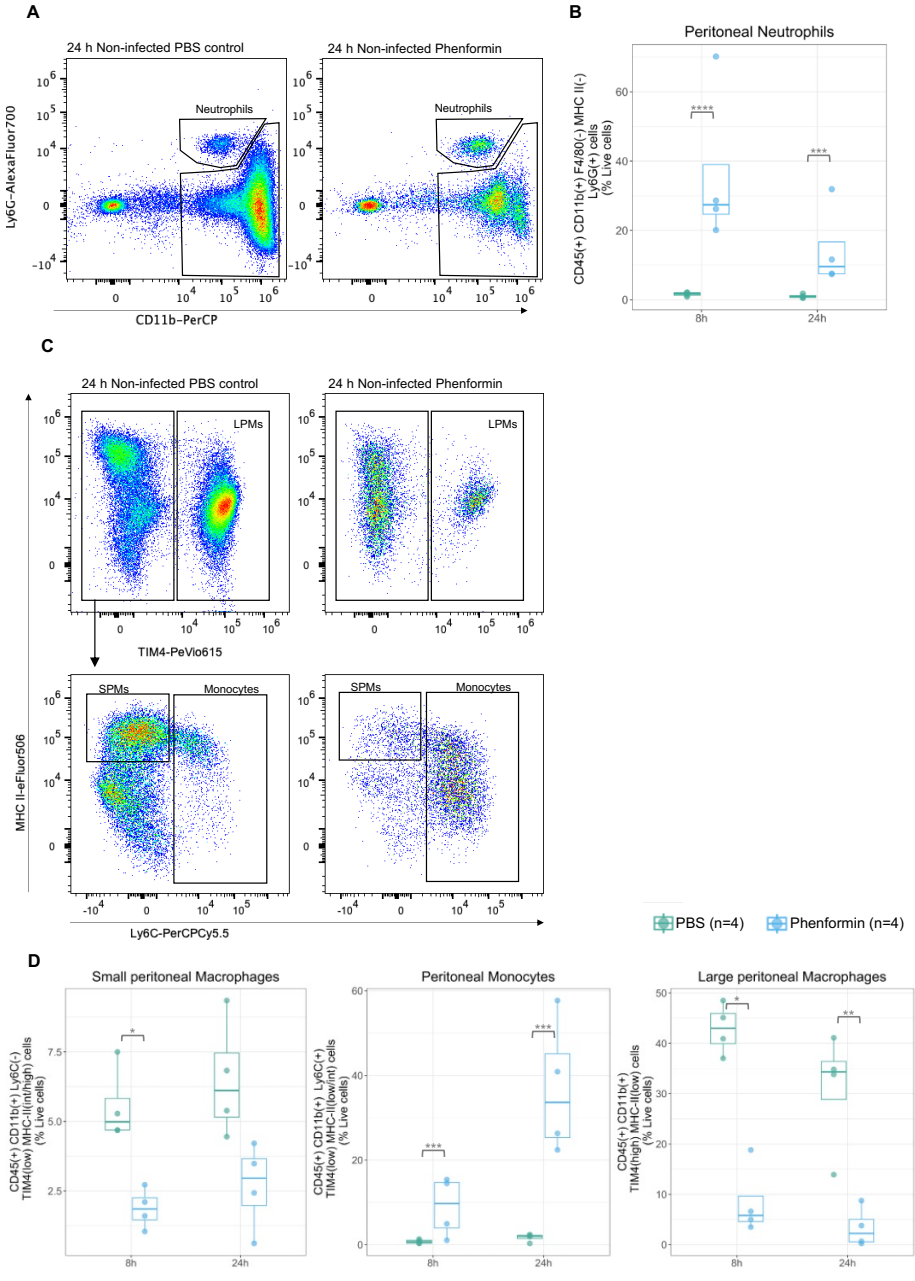

Figure 4. 8 - Neutrophil and monocyte phenformin-induced recruitment to the peritoneum.

Neutrophil, macrophage, and monocyte populations in the peritoneum of non-infected male C57BL/6J mice treated with 100 µg/g body weight phenformin (or PBS as a control). **(A)** Representative pseudocolor plots of LPMs (Live cells, CD45(+) CD11b(+) TIM4(+) MHC-II(low), SPMs (Live cells, CD45(+) CD11b(+) TIM4(-) MHC-II(int/high)) and monocytes (Live cells, CD45(+) CD11b(+) TIM4(-) MHC-II(low/int)) populations in peritoneum of vehicle control (left side) or phenformin treated (right side) mice at 24h post-injection. **(B)** Percentages (from left to right side) of small peritoneal macrophages (SPMs), peritoneal monocytes, and large peritoneal macrophages (LPMs) within live cells in different conditions and time points **(B)** represent pooled data of one experiment, individual mice measured are represented by points, and box plots indicate quartiles and median. Gating strategy can be found in Supplementary

Figure 4.4. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).

Together, these data indicate a modulating capacity of phenformin in the hematopoietic process which biases the bone marrow towards a reinforcement of myelopoiesis, a phenomenon described as emergency myelopoiesis[12].

4.4.5. BMDM functional capacity is not greatly increased by phenformin

Considering the results shown in Chapter 3, we have consistently observed an increase or faster recovery of macrophage populations induced by phenformin treatment during infection. In an attempt to understand whether phenformin could also modulate the functional capacity of macrophages in addition to the increased recruitment/differentiation, we have used an *in vitro* system. Here, we have co-cultured BMDMs with CFP expressing *E. coli* in the presence or absence of 100 μg/mL phenformin as section 4.3.2.8.. As a read-out of function, we analyzed the co-expression of CFP with macrophage markers by staining with fluorescently labeled antibodies at different time points after BMDM co-culture started. Results are shown in Figure 4.9.

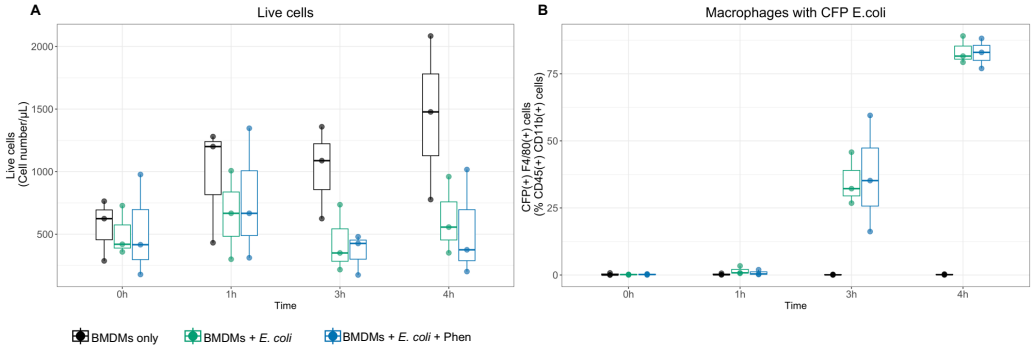

Figure 4. 9- Phenformin does not increase bone-marrow derived macrophage clearing capacity.

Bone-marrow derived macrophages were incubated with CFP-tagged StrepR *E. coli* at a ratio of one BMDM to two E. coli. Quantification of **(A)** live cell event count per μL of sample and **(B)** percentage of BMDMs with CFP fluorescence at indicated time-points in control conditions or in the presence of 100 μg/mL phenformin. **(A, B)** represent pooled data of three independent experiments, individual experiment measures are represented by points, and box plots indicate quartiles and median. **Statistical analysis** was done using R, by linear regression in logit-transformed data. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).

Data obtained so far does not seem to support an improved clearance capacity of *E. coli* by BMDMs exposed to phenformin.

4.5. Discussion

Aberrant lymphocyte responses are extensively described as being key players in the progression of conditions characterized by tissue damage and organ dysfunction. Autoimmunity, chronic hepatitis, and viral infections are among the many examples in which exacerbated response or over-activation of T lymphocytes and NK cells are at the root of the conditions[22,23]. Even in situations in which other immune cells are responsible for inducing injury, sustained cytokine signaling by T cell populations is an important driver by promoting inflammation, thus biasing other immune cells to support tissue damage profile[24,25]. A very interesting example of how T cells can indirectly cause tissue damage was reported in a study showing that CD8 cytotoxic T cells promote lung damage by inducing the expression of chemokine monocyte chemoattractant protein 1 (MCP-1) in alveolar cells infected with lytic virus. Herein, authors have shown that despite the short-lived presence of CD8 T cells in the lung, this interaction between the cytotoxic T cell and the infected alveolar cell produces a ripple effect that will promote extensive tissue damage several days after cytotoxic cell clearance from the lung, which results in infiltration of other mononucleated immune cells[26]. Other examples include the neutrophil NETosis process, which can be modulated by lymphocyte-derived cytokines, or even the polarization capacity of macrophages towards an anti-inflammatory or pro-inflammatory phenotype, which is also influenced by the cytokines these cells are exposed to in their environment[27–29]. Thus, the balance of T lymphocyte populations and their activation status does not only affect possible tissue damage events directly but also through modulation of other immune cell populations.

Sepsis is an immune-driven condition that results from a primary hyperactive immune response[30]. This leads to extensive tissue damage driven by cytokine storm and overactivation of pro-inflammatory profiles in several immune cell types, followed by immune paralysis phase, characterized by the exhaustion of these cell populations and a lack of capacity to promote tissue repair mechanisms[30]. In Chapter 3, we described that mild mitochondrial stress, induced via a single administration of phenformin, reduces overall lymphocyte and NK cell expression of the surface activation marker CD69. Despite its classical connotation to activation, CD69 has been described to have many more roles in T and NK cell maturation, development, and functional capacities. Thus, we aimed to understand the importance of this downregulation of CD69 on sepsis progression and tolerance mechanisms in our murine mouse model with the ultimate goal of understanding whether control of T and NK cell responses through downregulation of CD69 could be the mechanisms by which phenformin is inducing disease tolerance and promoting the tissue protection, as described in Chapter 2. So far, we have

tested whether downregulation of CD69 could be (1) impacting T regulatory cells (Tregs) differentiation and (2) reducing T cell-driven damage.

We had hypothesized that, since CD69 is involved in the fate decision of T cell differentiation into Th17 or Treg, phenformin might be modulating T cell differentiation towards a regulatory profile. This could ultimately induce protection by promoting the increase of suppressive Treg cells that might exert control over the remaining T cell populations and prevent an overly exacerbated inflammatory response. In fact, other studies have shown that complex I of the mitochondrial ETC is fundamental for proliferation and T cell activation[16], and co-administration of tacrolimus and metformin showed increased T regulatory and decreased Th17 cells in solid organ transplant patients[21]. However, we did not find any significant impact in splenocyte Treg populations associated with phenformin treatment of septic mice. This could indicate that CD69 downregulation in these cells is not impacting their fate decision and that an alternative process driven by this marker might have a more important role, such as modulating tissue egress and lymphoid organ homing. Admittedly, we have only assessed this at 24 h after infection and treatment and only in the spleen. Considering the classical CD4 T cell responses, this might be a caveat of our experimental approach and represent the incorrect time point or organ for us to see a difference in the profile of CD4 cell populations induced by phenformin.

Another immune population that showed a considerable reduction of CD69 marker with phenformin treatment was the cytotoxic CD8 T cells. Unlike CD4 T cells, cytotoxic CD8 T cells have a much faster response time, and CD69 expression is highly connected to their cytotoxic capacity[31]. Thus, we aimed to understand whether phenformin's downregulation of CD69 on CD8 T cells controlled the cytotoxic profile of this cell population and, in this way, reduced tissue damage. Our CD8 T splenocyte cell transfer experiments did not yield supportive results for this hypothesis. In fact, the level of tissue damage we observed in recipient mice was neither significantly elevated nor different between groups. One reason for this might again lie with the time point selected for this experimental layout since the recipient cells transferred cannot exert sufficient damage within 24 h to be measurable. Another important and possible reason for not detecting differences might be related to the fact that recipients are naïve mice that never underwent an infection nor did after the transfer. In fact, sepsis as a multifactorial and systemic condition, is the result of several parallel events. Thus, it is not surprising that simply transferring lesser or more activated CD8 cytotoxic T cells (or whole splenocytes) is not enough to phenocopy the tissue damage in the recipients and the consequent protection conferred by phenformin.

Considering this, we have resorted to an alternative approach by removing lymphocytes instead of transferring them into naïve mice, using the B6.Rag2 KO mouse model. Remarkably, removing lymphocyte populations from an organism did not impact phenformin's protective capacity during infection. B6.Rag2 KO mice showed very similar disease severity to wild-type animals, circulating damage marker levels, and CFUs. In fact, AST, ALT, and LDH reduction with phenformin treatment and pathogen clearance kinetics are extremely similar. This could indicate that the downregulation of CD69 in CD4, CD8, and B cells might not be an underlying factor of the lower tissue damage scores and circulating damage markers observed in phenformin-treated animals.

Nonetheless, a major caveat of using B6.Rag2 KO mice is that these animals have increased numbers of highly activated cytotoxic NK cells[32,33]. Hence, using them as a model to test whether phenformin downregulation of CD69 on lymphocyte populations is the driver of disease tolerance mechanisms that protect mice from sepsis might not be ideal since (1) we have also found that phenformin reduces CD69 in NK cells. Thus, this might be a phenotype that arises from the control of the exacerbated response of several cell populations and (2) as these mice already have naturally more activated, cytotoxic, and increased numbers of NK cells, we cannot extrapolate that phenformin's efficiency in downregulating CD69 will be the same as in wild-type animals with lower baseline activation levels.

Another important finding discussed in Chapter 3 of this work was the increased recruitment of innate immune cells to the infection induction site, as well as the increased overall viability and cellularity in phenformin-treated mice. Taken together with the remarkable impact of phenformin during homeostasis on peritoneal cavity populations, which we suspect is an indication of Macrophage Disturbance of Homeostasis Reaction, this led us to hypothesize that phenformin could be modulating hematopoiesis.

Hematopoiesis is a highly controlled process that occurs in the bone marrow, and that an organism can use to replenish immune cells adequately. Classically, it was believed that monocytes are differentiated from GMPs that stem from Flt3(-) CMPs. However, studies have shown that stress can stimulate the bypass of this conventional differentiation trajectory and allow for an independent lineage differentiation trajectory for monocytes through Flt3(+) CMPs monocyte-dendritic cell progenitors (MDPs). Herein, the former monocytes differentiate into monocyte-derived dendritic cells, whereas the monocytes arising from MDPs have a "neutrophil-like" profile[34,35]. To understand the possible impact of phenformin in modulating the hematopoietic process we have characterized bone marrow progenitor and peritoneal cavity populations at 8 and 24 h after phenformin treatment. Our results support that phenformin could modulate the hematopoietic process towards an emergency myelopoiesis phenotype.

We observe a reduction of LT-HSC and MPP3 already at 8 h post-injection, which we speculate to be a result of the faster differentiation rate of Flt3(+) CMPs and MDPs and the increase of GPs and cMoPs. At 24 h after phenformin injection, LT-HSC have recovered to homeostatic levels, and MPPs seem to be increased, possibly resulting from compensatory mechanisms to establish homeostatic levels after the high demand of differentiation these progenitors have faced due to phenformin injection. Interestingly, bulk GMPs and GPs still show increased levels at 24 h after phenformin injection, indicating that phenformin modulates the hematopoietic process at least up until 24 h. In line with this, we see a strong recruitment of neutrophils and monocytes in the peritoneal cavity that is observable and maintained during 24 h, which we speculate results of this phenformin-driven reinforcement in myelopoiesis in the bone marrow.

Altogether, these data suggest that phenformin has a modulatory capacity of myeloid progenitors that will reinforce the differentiation of neutrophils and monocytes even without infection. Considering previous work by Mitroulis *et al.* and Zahalka *et al*, where authors have shown the importance and potential of modulating myeloid progenitors in the resolution of localized and systemic inflammation[13,36], we can speculate that phenformin might be eliciting similar mechanisms and priming the innate immune system which ultimately, during infection, results in a more efficient and faster infection resolution capacity of the host.

Acknowledgments

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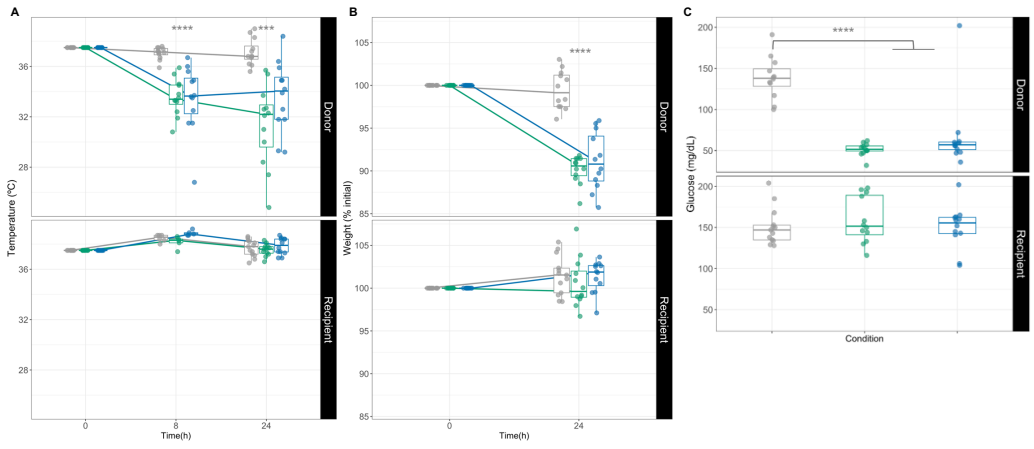
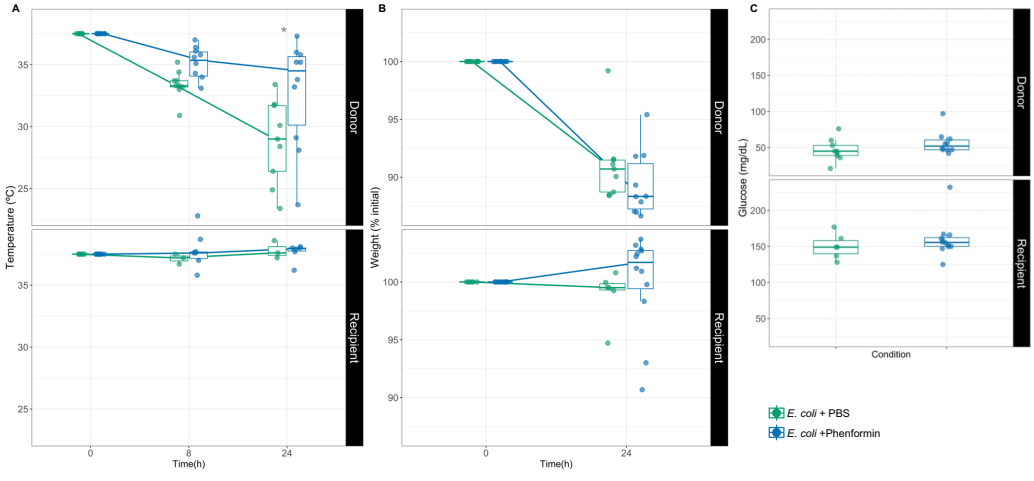
4.7. Supplementary data



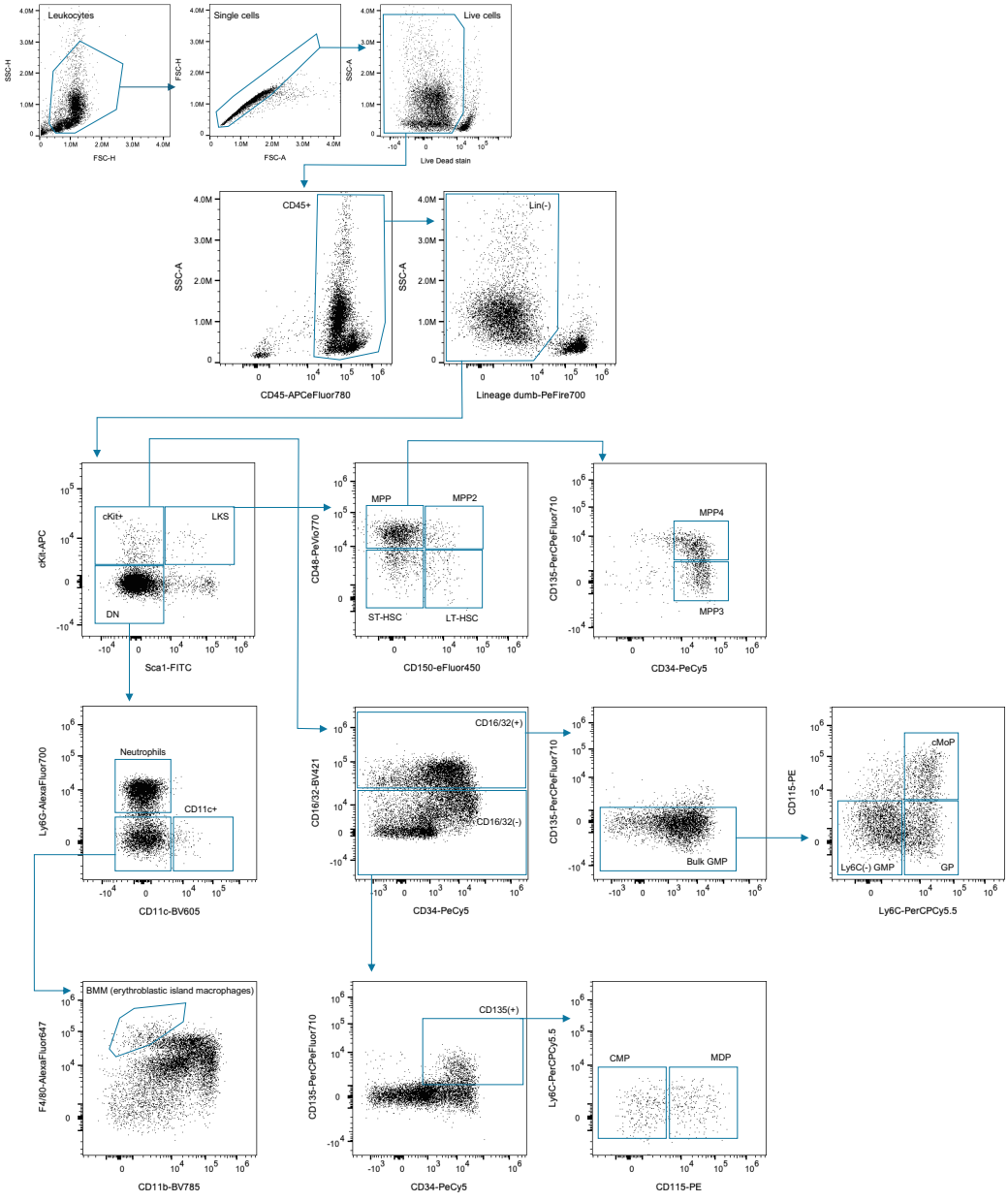
Supplementary Figure 4. 1 - Vital parameter from adoptive cell transfer experiments of whole spleen.

Rectal temperature **(A),** % initial body weight **(B)**, and glucose **(C)**, after infection of male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) for donor mice, or after whole splenocyte cell transfer for recipient mice, at 0, 24 h. Plots represent pooled data from three independent experiments, individual mice measured are represented by points, and box plots indicate quartiles and median.. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time points independent of experiment. Temperature, glucose, and weight were analyzed by linear regression. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).

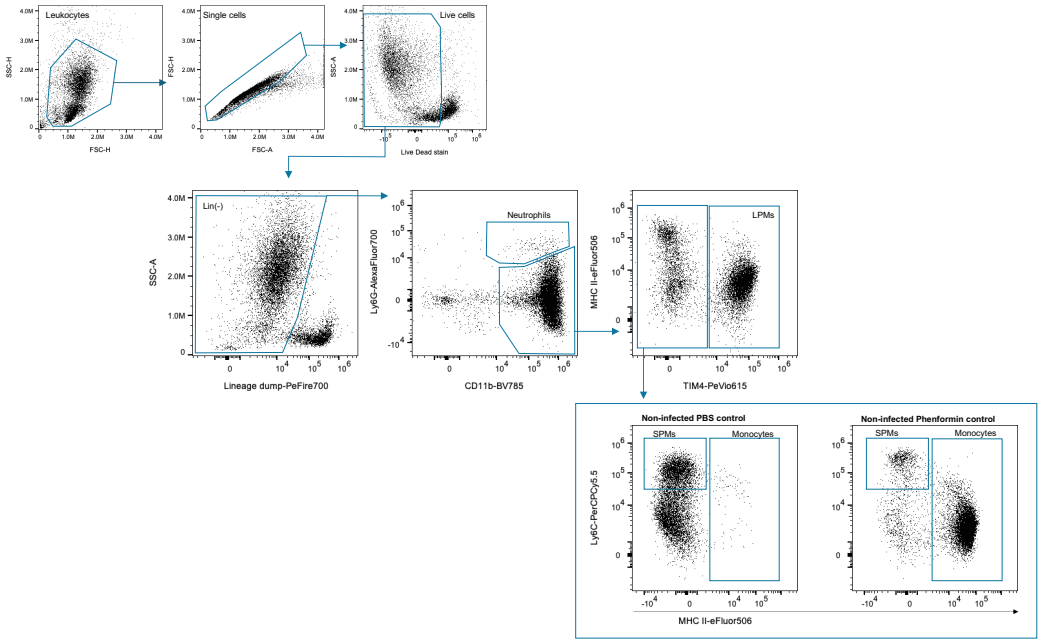


Supplementary Figure 4. 2 - Vital parameter from adoptive cell transfer experiments of CD8 T lymphocytes.

Rectal temperature **(A),** % initial body weight **(B)**, and glucose **(C)**, after infection of male C57BL/6J mice with 3 - 4 x 10^8 CFU/mouse StrepR *E. coli* and treatment with 100 µg/g body weight phenformin (or PBS as a control) for donor mice, or after splenocyte CD8 cell transfer (recipients) at 0, 24 h. Plots represent pooled data from three independent experiments. **Statistical analysis** was done using R, by modeling the effect of treatment across the different time-points independent of experiment, individual mice measured are represented by points, and box plots indicate quartiles and median. Temperature, glucose, and weight were analyzed by linear regression. The following symbol indicates statistical significance: p < 0.05 (*), p<0.01 (**), p<0.001 (***), p<0.0001(****).



Supplementary Figure 4. 3 - Gating strategy of bone marrow myeloid and lymphoid progenitors and fully committed cells.



Supplementary Figure 4. 4 - Gating strategy of peritoneal lavage fluid neutrophils, dendritic cells, small peritoneal macrophages, large peritoneal macrophages and monocytes.

Chapter 5

General discussion and future perspectives

The capacity to maintain organismal equilibrium independently of external changes, described in the literature as homeostasis, has been the subject of extensive study since its conception. Understanding how individual cells and even whole organisms maintain their biological processes intact upon challenges and what are the thresholds of endurance to stressors before becoming deleterious could help elucidate the fundamentals of organismal biology and the underlying mechanisms for the development of chronic pathological conditions. In particular, the ability of cells and organisms to adapt to stressors and restore homeostasis after an insult, also known as hormesis, has sparked great interest within different fields of biology. From the pioneering work conducted with *C. elegans*, demonstrating the potential of inducing stress in core cellular functions to harness protective mechanisms and even lifespan expansion, to the growing number of studies in mouse models corroborating that these are evolutionarily conserved mechanisms[1–4]. The research field of hormesis has been accumulating irrefutable evidence that stress responses are critical regulators of cellular and organismal homeostasis, with important roles not only in surveillance immunity but also in the orchestrations of responses and adaptations to challenges.

The host laboratory has been at the forefront of demonstrating the role of stress responses in mouse models of infection. This is of great relevance for understanding to which extent these mechanisms are conserved between simpler organisms, such as the nematode *C. elegans*, and mammals, but also to dissect the potential of using stress to promote hormesis and harness protective mechanisms in conditions of extreme organismal dysregulation, such as sepsis. In line with this, we have thus far demonstrated that DNA damage responses and mild mitochondria stress elicit protective mechanisms during sepsis, with treated mice having improved survival to the severe infection with lower tissue damage scores, independently of pathogen load[3,5]. Other groups have since validated that mitochondrial stress can be protective in different infection models or during other conditions[4,6,7].

Considering the diversity of functional responsibilities that mitochondria fulfill within a cell and in cell-to-cell communication, this thesis focused on further exploring and elucidating the physiological impact of inducing mild mitochondrial stress during infection, using sepsis as a model for extreme organismal dysregulation. Our previous work demonstrated that different drugs targeting different subunits of the mitochondrial respiratory chain could elicit disease tolerance mechanisms and protect severely septic mice, improving survival and decreasing organ damage independently of the pathogen burden. Our previous work mainly focused on the effect of tetracyclines, which inhibit complexes III and IV of the ETC. However, it also highlighted the striking protective potential of phenformin, a complex I inhibitor[3]. Phenformin treatment elicited the strongest protective phenotype of all tested drugs. Interestingly, the

protection conferred by phenformin was also significantly greater than metformin. Thus, the current work focused on exploring the effect of phenformin, as a complex I inhibitor, in protecting mice from sepsis.

In addition to focusing on one specific mitochondrial stressor (complex I of the ETC inhibition by administration of phenformin), this work also aimed at elucidating the contribution of tolerance and resistance mechanisms in the protection conferred to sepsis. Our previous work described the protective mechanisms elicited by mild mitochondrial stress within a very short period of time in a very severe model of sepsis. To fully understand the cellular and physiological changes elicited by phenformin treatment of septic mice, this current work resorted to an optimized model of sepsis that induces a milder infection. This has allowed us to follow the mice for longer timeframes, ultimately providing a framework for the dissection of the protective mechanisms of mild mitochondrial stress in finer detail.

In line with our previous results, we have found that mild mitochondria stress, induced by phenformin treatment, protects mice from mild sepsis, promoting a faster and more efficient recovery from the infection. This is demonstrated by the physiological recovery of temperature, blood glucose levels, and weight. As described in Colaço *et al.*[3], this protection is, during the first stage, conferred by lower inflammation and reduced tissue damage, independently of pathogen burden, thus resulting from disease tolerance mechanisms. Interestingly, this milder sepsis model allowed us to observe that during later stages, the host is capable of switching from tolerance towards resistance phenotypes. This is demonstrated by the increased efficiency in pathogen clearance and infection resolution of phenformin-treated mice. These results suggest that using the severe model of sepsis limited the temporal window for characterization to the full extent of the protective mechanisms elicited by phenformin. With this milder model of sepsis, we now have a platform to dissect in finer detail the full array of protective mechanisms of phenformin during bacterial infection.

To understand cellular changes induced by phenformin treatment of septic mice that promote tolerance and resistance mechanisms, we characterized the immune cell populations across tissues throughout infection. This has provided evidence that the same stress has different impacts depending on the cell type. In detail, our results showed that phenformin treatment reduces activation of lymphocyte and NK cell populations, while in parallel, promoting faster recruitment of neutrophils, monocytes, and macrophages to the site of infection initiation. Interestingly, within the peritoneal cavity, phenformin treatment triggers Macrophage Disturbance of Homeostasis Reaction (MDHR) (even without infection), a phenomenon demonstrated to occur and play a crucial role in *E. coli* infection and clearance[8,9]. This, in turn,

results in monocyte recruitment to the peritoneal cavity, where we speculate that these cells will differentiate and commit to becoming tissue-resident macrophages.

Despite not being reported yet in an integrated fashion in the literature, many studies describe the heterogenous role that mitochondria can have within different cells and tissues. Perhaps, the simplest explanation for this relies on the fact that distinct immune cells have different bioenergetic requirements due to their core functional programs. Thus, inhibition of complex I of the ETC might exert various effects depending on whether cells rely to a vast extent on OXPHOS for energy production or rather utilize mostly glycolysis to fulfill their energetic needs.

This has been nicely demonstrated in different studies showing that T helper cells switch from OXPHOS to glycolysis upon stimulation to allow for proliferation and effector function, whereas T regulatory cells maintain as their energetic source OXPHOS, except during migration[10–12]. Taken together with the observation that metformin co-administration with tacrolimus can promote the expansion of Treg cells, we could speculate that the inhibition of complex I induced by metformin (in similarity to phenformin) is promoting bioenergetic profiles that support regulatory cell differentiation in detriment of T helper cell[13]. Additionally, several independent research groups have reported that ETC complex I inhibition attenuates T cell activation and modulates proliferation[14,15]. In line with this, our results show that phenformin treatment reduces CD69 expression in lymphocyte and NK cell populations.

To disentangle the impact of ETC complex I inhibition in NK cells could represent a bigger challenge. This is because these cells share functional similarities with both, innate and adaptive immune cells. It is known that NK cells can rely on both OXPHOS and glycolysis to fulfill their energetic requirement[16]. Interestingly, recent studies suggest that mitochondrial stress results in impaired activation and cytotoxic capacity[17–19]. Thus, just as for T cells, we can speculate that mitochondria stress targeting complex I of the ETC could be controlling the exacerbation of NK cell responses.

Innate immune cells can also be highly influenced by changes in their energy sources. Neutrophils rely to the vast majority on the glycolysis process for their energy demands, thus compromising ETC complex I of the mitochondria has been reported to hamper neutrophil effector function, interestingly, other studies have shown that mitochondrial dysregulation can promote inflammatory diseases[20,21]. Similarly, macrophages can be metabolically reprogrammed to pro-inflammatory phenotypes by promotion of glycolysis, on the other hand, oxidative mitochondrial stress can control inflammatory responses during macrophage activation[22,23]. The literature on the role of the ETC complex I in monocytes is still scarce and incomplete, however, considering the functional responsibility these cells fulfill in differentiating

into macrophages and dendritic cells upon stimulation, this indicates a high dependency on energetic availability for metabolic rewiring, and proper effector function. Thus, it is not illogical to speculate that inhibition of complex I of the mitochondria can reprogram monocytes and influence these cells' proliferative capacity[24]. Furthermore, monocyte recruitment and proliferation results from a modulation of hematopoietic cell differentiation process to adapt to a higher demand, thus we could speculate that the changes within the monocyte population might reflect a modulation of the process of hematopoiesis[25,26].

Considering our results and what has been reported so far in the literature, we hypothesized that:

(1) the disease tolerance mechanisms elicited by phenformin during sepsis are dependent on lymphocyte and NK cell downregulation of CD69, which could have roles in attenuating T cell responses and proliferation, promoting a regulatory profile in cells and controlling effector cytotoxic function of CD8 T and NK cells;

(2) the disease resistance mechanisms result from the more efficient and preferential recruitment of innate immune cells to the site of infection initiation, together with the role of phenformin in inducing MDHR and monocyte recruitment to the peritoneum, which in turn can pre-emptively activate myeloid cell differentiation.

Notwithstanding the growing body of evidence, attempting to elucidate the different roles and the impact of mitochondrial stress in cells, studies are not systemic, and the observations do not allow for an integrated review of the effect of specific mitochondrial stresses in different cells within a whole organism. Thus, we sought to address these questions, by validating the role of lymphocytes to exert tissue damage and by investigating the role of phenformin in promoting myelopoiesis.

Despite our best efforts, the connection between the reduction of CD69 on lymphocyte and NK cell populations and reduced tissue damage associated with phenformin treatment of septic mice remains elusive. Our initial hypothesis suggests that downregulation and control of lymphocyte and NK cell populations' inflammatory response could be the underlying mechanism of the phenformin-induced disease tolerance. However, we have yet to find the appropriate experimental setting to validate this thoroughly. The experiments performed so far characterized poorly in a rather simplified system with non-negligible caveats some of the

possible effects of downregulating CD69 in certain populations. Additionally, we cannot disregard that the tissue protection phenotype we observe with phenformin treatment of septic mice might result from several isolated consequences of phenformin on different cell subsets that only become significant when the effect is systemic. For example, it is possible that CD69 downregulation might be fundamental for tissue protection due to its capacity to control inflammatory responses of T cells and the cytotoxic reaction of CD8 T and NK cells not only due to its direct cellular effect but also through the modulation of cytokine secretion of these cells that could be involved in reducing the influx of neutrophils and monocytes in the lung. Ultimately, these two events might be connected and, consequently lead to the lower lung inflammation reported in this work.

On the other hand, we have demonstrated that phenformin induces emergency myelopoiesis and promotes the recruitment of neutrophils and monocytes to the peritoneal cavity, even in the absence of infection. Our results suggest that phenformin-induced mild mitochondrial stress modulates the hematopoietic process, driving the prioritization of myeloid lineage differentiation. This promotes a significant recruitment of neutrophils and monocytes to the peritoneal cavity. Other authors have reported similar results as a consequence of infection or other stimuli[8,27–29]. We speculate that incoming peritoneal monocytes, differentiate into fully committed tissue-resident macrophages. Ultimately, the pre-emptive recruitment of monocytes into the peritoneal cavity and accelerated tissue-resident macrophage population replenishment could protect by providing more efficient pathogen clearance and infection resolution.

Figure 5.1 illustrates the key findings of this work and attempts to conceptualize a working model for the protective effects harnessed by phenformin, upon which research questions can be built to address this project's remaining knowledge gaps. Taken together, the results gathered within the scope of this work have allowed us to demonstrate that phenformin-induced mild mitochondrial stress can promote protective mechanisms that improve the survival and disease recovery of severely and mild septic mice. This protection is conferred by the preferential promotion of disease tolerance mechanisms during the early stages of infection, followed by a switch in the main host defense program towards promoting resistance and pathogen clearance responses. In detail, we have reported evidence that mild mitochondrial stress by phenformin administration impacts the organism in a cell- and organ-specific manner. We speculate that within lymphocyte and NK cells, stress is hampering the proliferation and activation mechanisms of these cells, ultimately compromising the effector function in the early stages of infection; this in turn, might prevent the exacerbation of

responses of these types of cells, which is highly associated with immune paralysis because of exhaustion resulting for the extreme demand of effector functions conditioned by early activation. On the other hand, the capacity of phenformin to modulate hematopoiesis and promote emergency myelopoiesis, even without infection, provides a compelling argument for improved resistance responses during infection, as this will result in pre-emptive recruitment of neutrophils as first responders to infection, and monocytes as precursors of tissue-resident macrophages, and key players in bacterial clearance.

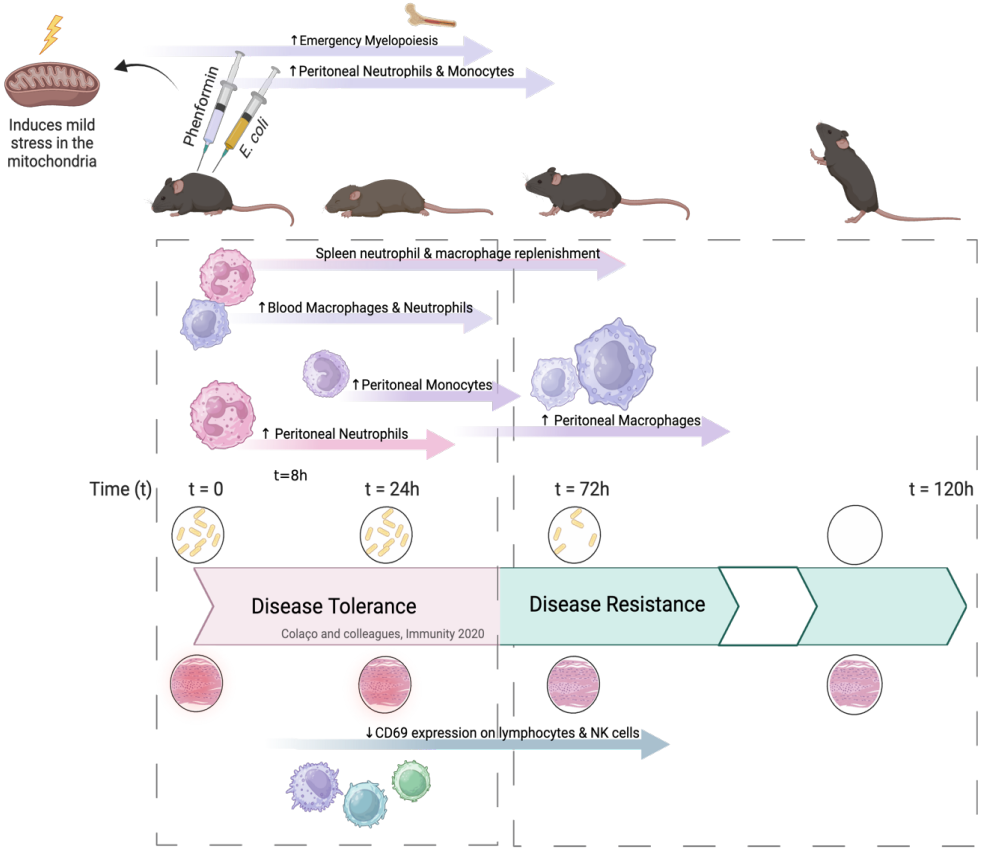


Figure 5. 1 – Mechanisms of protection conferred by mild mitochondrial stress during infection.

Working model illustrating the disease tolerance and resistance mechanisms induced by administration of phenformin to septic mice. Original illustration created in Biorender.

Henceforth, it will be important to clarify the validity of these hypotheses by dissecting (1) the role of CD69 downregulation in disease tolerance mechanism and in the reduction of inflammation and tissue damage and (2) the relevance of the emergency myelopoiesis in resistance mechanisms for infection clearance.

As was mentioned before, attempts at understanding the biological relevance of the downregulation of CD69 in lymphocytes have failed to demonstrate any correlation with lower damage scores, and disease tolerance mechanisms. This might indicate that the decreased CD69 expression in lymphocytes does not play a role in tissue damage reduction or, as also suggested, that the experimental design has major caveats that prevented the demonstration of a possible connection. Thus, it might be beneficial to re-evaluate cytotoxic CD8 T cell ability to induce damage by performing adoptive cell transfers into non-naïve animals or by assessing the damage after longer periods of transferred-cell exposure. On the other hand, we have not yet addressed the question of the role of phenformin-driven CD69 reduction on NK cells in preventing tissue damage.

In fact, literature reports that NK cells are fundamental players in the orchestration of inflammatory responses during bacterial sepsis and that the immune suppression of these cells is often observed during sepsis, and associated with immune paralysis[30,31]. Thus, it becomes highly appealing to investigate the role of CD69 downregulation in NK cells, exploring the previously listed hypotheses. For one, reduction of CD69 in NK cells early on during infection might prevent the overactivation of these cells, in turn preventing exhaustion and immune paralysis. Moreover, this control of NK cell response might have a broader effect during sepsis recovery, since preventing NK cell exhaustion from extreme early-on activation might allow proper orchestration of pathogen clearance mechanisms by these cells at later time points. To investigate these hypotheses, studies with adoptive cell transfer of negatively isolated NK cells from the infected vehicle- and phenformin-treated mice could be performed alongside NK cell depletion via monoclonal antibody injection, and infection characterization. This would allow us to understand whether NK cells can induce damage in recipient mice and whether phenformin-driven CD69 reduction could reduce damage promoted by these cells. In parallel, depleting NK cells would provide evidence for the extent of tissue damage driven by NK cell responses during our bacterial sepsis model. Lastly, it could be interesting to use a mouse model that does not have lymphoid cells, such as an NSG™ mouse. In line with the experiments with B6.Rag2 KO mice already conducted, this would allow for clarification of T, B, and NK cell role in inducing tissue damage during bacterial sepsis, with the surplus that this model does not have naturally activated NK cells, ultimately addressing one of the caveats exposed from the experiments conducted so far[32].

After identifying the cell(s) subsets involved in inducing tissue damage during sepsis, it would also be important to understand in detail what the consequences of CD69 reduction represent in each specific cell subset. Despite being canonically described as an early activation marker, the roles of CD69 greatly exceed this function. With reports showing involvement in cell

proliferation, migration, and even effector function[33]. Thus, understanding the role of phenformin-driven reduction of CD69 in different cell subsets could elucidate the mechanisms by which it confers protection. For this, strategies of finer immunophenotyping across tissues could be applied to identify different cellular subsets of T and NK cells, allowing for the dissection of the impact on cell differentiation processes. Herein, it would be relevant to confirm the previously described results gathered for CD8 T cytotoxic and CD4 T helper cells by adding CD3 as an extra identification marker for proper T lymphocytes. For effector function assessment, additional *in vitro* degranulation assays could be performed to evaluate NK cell cytotoxic function in the presence or absence of phenformin. A similar approach could be used for CD8 T cytotoxic cells.

On the other hand, despite having a better understanding of how resistance mechanisms might be promoted by phenformin, results gathered so far are only correlational, and a deeper elucidation of the biological relevance of population dynamic changes is needed. Herein, it would be necessary to first clarify whether the modulation of the hematopoietic process occurs as a direct consequence of phenformin-induced stress on the bone marrow or as a result of the MDHR-induced in the peritoneal cavity. Hereafter, validation that the peritoneal incoming neutrophils and monocytes are deriving from bone marrow differentiation would be required. For this, a mouse model of induced fluorescence in bone marrow progenitors under exposure to phenformin could be used to trace bone marrow-derived cells across tissues. Furthermore, the depth of the impact of modulation of the hematopoietic process should be investigated to understand whether these changes are being imprinted into the progenitors by metabolic reprogramming or epigenetic changes, which in the long term would provide trained immunity protection mechanisms[28,29,34,35]. In addition to understanding the mechanisms by which phenformin can modulate hematopoiesis and increase innate immune cell recruitment, it is also important to validate that these are the mechanisms and the cell population responsible for the improved pathogen clearance phenotype. Thus, the capacity of monocytes to differentiate into tissue-resident macrophages and the ability of these cells to target the invading pathogen and promote clearing mechanisms should be subject to further investigation. Herein, a more comprehensive understanding of the ontology of the monocytes recruited to different tissues by phenformin could help elucidate their role in tolerance and resistance to infection[26].

In addition to the impact of phenformin treatment in modulating mitochondrial respiration and cellular bioenergetic dynamics, the inhibition of complex I could promote the release of mitochondria-derived signaling molecules, which have been extensively reported to have a fundamental role in cell signaling and inflammation processes[36]. Since the first studies

demonstrating the existence and role of mitokines in intra- and extracellular signaling, it has become clear that even when targeted to the electron transport chain, stressors of the mitochondria can promote the release of danger signaling molecules that will elicit cellular responses. Because of this, it would ultimately be important to understand whether the protective phenotypes we have elicited with phenformin administration are a consequence of the complex I inhibition and the direct modulation and adaptation of energetic and metabolic cellular processes or if these result from mitochondrial-derived signaling molecules, or a combination of both. This could potentially improve host response modulation strategies.

Overall, the work developed under the scope of this thesis has provided evidence for the role of mild mitochondrial stress in inducing hormesis and promoting organismal adaptation and protection against infection. The changes in cellular dynamics reported here allow us to formulate hypotheses and speculate on the biological relevance of different cell populations in the development of chronic inflammation and in promoting effective pathogen clearance and inflammation resolution. This knowledge adds to the increasing evidence demonstrating that mitochondria function as central orchestration hubs for host-pathogen interactions and as crucial players in both surveillance and response mechanisms of organismal defense systems.

More than the direct implication in bacterial sepsis, the findings reported here could potentially have applications in inflammatory conditions and even in other complex conditions such as autoimmunity or cancer, as they provide a window into the mechanistic basis by which resistance and tolerance mechanisms can be harnessed. Naturally, the extrapolation of these findings into different scenarios and in understanding the clinical applicability requires further experimental validation, using different models of infection (viral and fungal infection for example) and different experimental animal models, including different strains and possibly outbred mice models. If encouraging results are accumulated with these experimental approaches, validation in larger mammals, such as pigs, should be considered.

The quest of the search for an elixir of life has been around since 120 B.C., when King Mithridates VI, of Pontus, pursued protection from poisoning by ingesting small doses of a variety of venoms[37]. About 500 years ago, Paracelsus put these actions into a framework by describing that “all things are poison, and nothing is without poison. Solely the dose determines that a thing is not a poison”[38]. Even though the concept of hormesis, was only coined in 1943 by Southam and Ehrlich, evidence for the biological relevance of this is abundant and highly representative across evolution and species[39,40]. Indeed, a growing

number of studies strongly support the potential of understanding the mechanisms by which stress can induce the adaptation of organisms, for the development of better strategies to promote long-term protection by an improved balance between resistance and tolerance mechanisms. This might ultimately represent a fundamental cornerstone in the development of therapeutic strategies to tackle the challenges we are facing in biology and modern medicine.

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Appendix

(1) **Tetracyclines: four rings to rule infections through resistance and disease tolerance**

Kátia Jesus, Luís F. Moita

J Clin Invest. 2022;132(17):e162331.

https://doi.org/10.1172/JCI162331.

(2) **Ketogenesis favors oxidative phosphorylation to promote disease tolerance**

Kátia Jesus, Luís F. Moita

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Commentary

Several classes of antibiotics have long been known for protective properties that cannot be explained through their direct antimicrobial effects. However, the molecular bases of these beneficial roles have been elusive. In this issue of the *JCI*, Mottis et al. report that tetracyclines induced disease tolerance against influenza virus infection, expanding their protection potential beyond resistance and disease tolerance against bacterial infections. The authors dissociated tetracycline's disease-resistance properties from its disease-tolerance properties by identifying potent tetracycline derivatives with minimal antimicrobial activity but increased capacity to induce an adaptive mitochondrial stress response that initiated disease tolerance mechanisms. These findings have potential clinical applications in viral infections.

Tetracyclines: four rings to rule infections through resistance and disease tolerance

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Several classes of antibiotics have long been known for protective properties that cannot be explained through their direct antimicrobial effects. However, the molecular bases of these beneficial roles have been elusive. In this issue of the *JCI*, Mottis et al. report that tetracyclines induced disease tolerance against influenza virus infection, expanding their protection potential beyond resistance and disease tolerance against bacterial infections. The authors dissociated tetracycline's disease-resistance properties from its disease-tolerance properties by identifying potent tetracycline derivatives with minimal antimicrobial activity but increased capacity to induce an adaptive mitochondrial stress response that initiated disease tolerance mechanisms. These findings have potential clinical applications in viral infections.

Strategies to survive severe infection

ICU patients with sepsis often present dramatically different outcomes despite having had a similar initiating pathogen or pathogen load, or even having completely eliminated the original infection. The contrasting outcomes may be explained by the need for two different but interdependent and evolutionarily conserved defense strategies to survive a severe infection: resistance, which relies on effector mechanisms to reduce pathogen load, and disease tolerance, which provides host tissue damage control and limits disease severity irrespective of pathogen load (1). Research on the initiation of protective immune responses has so far mostly focused on the direct sensing of microorganisms via pattern recognition receptors (PRRs). The pattern-triggered immunity model (2) states that PRRs recognize microorganism-associated molecular patterns (MAMPs) representative of different groups of microorganisms, which leads to the activation of effector mechanisms adjusted to each pathogen group. This model is well supported by data but fails to explain how the host can respond to pathogens with which it has no evolutionary history (3). Critically, the model is insufficient to explain how vertebrate hosts discriminate between commensal and pathogenic microorganisms that display similar MAMPs. While much progress has been made as to which and how immune circuits sense different groups of pathogens, current models still lack a comprehensive conceptual framework for immune responses. For example, the danger model (4) values contextual cues of pathogen-induced damage-associated molecular patterns but poorly explains the initiation step and has not resolved its mechanistic inconsistencies (5). Alternatively, the effector-triggered immunity (6) model proposes that the immune system recognizes pathogens by sensing virulence factors or activities (7, 8), but does not account for substantial cellular physiological perturbations that are not caused by the direct or indirect effects of virulence factors used by pathogens. It is likely that in addition to directly recognizing conserved microorganism molecular signatures using PRRs, the host mounts an immune response after sensing a homeostatic disruption that serves as a proximal reporter for infections (9). Interestingly, several groups of pathogens, including viruses and bacteria, target and perturb different organelles, including mitochondria (10). The host's ability to sense a homeostatic disruption may be a key component for detecting the presence of a disease-causing microorganism. These pathways may synergize with the sensing capability of PRRs not only to potentiate the resulting feed-forward mechanisms that contribute to initiating the immune response, but also to inform the host on the intensity of the threat posed by the pathogen. The early events triggered by a disruption of homeostasis may also have a role in limiting tissue damage and in later negative-feedback pathways that terminate the inflammatory response and activate tissue repair, allowing for a full return to steady state.

Antibiotics have effects beyond their direct antimicrobial activities

Physicians have known, and empirically used for decades, several classes of antibiotics that seem to better resolve an infection than would be expected from their direct antimicrobial efficacy alone, comparing favorably with other classes that have similar antimicrobial spectra. In addition, for example, macrolides have extensively documented clinically beneficial roles in chronic inflammatory pulmonary disorders (11, 12), and demonstrated protective effects in models of cerebral ischemia (13). Other classes, including fluoroquinolones and tetracyclines, have also been vaguely labeled as immunomodulators, but the molecular mechanistic bases remain unidentified

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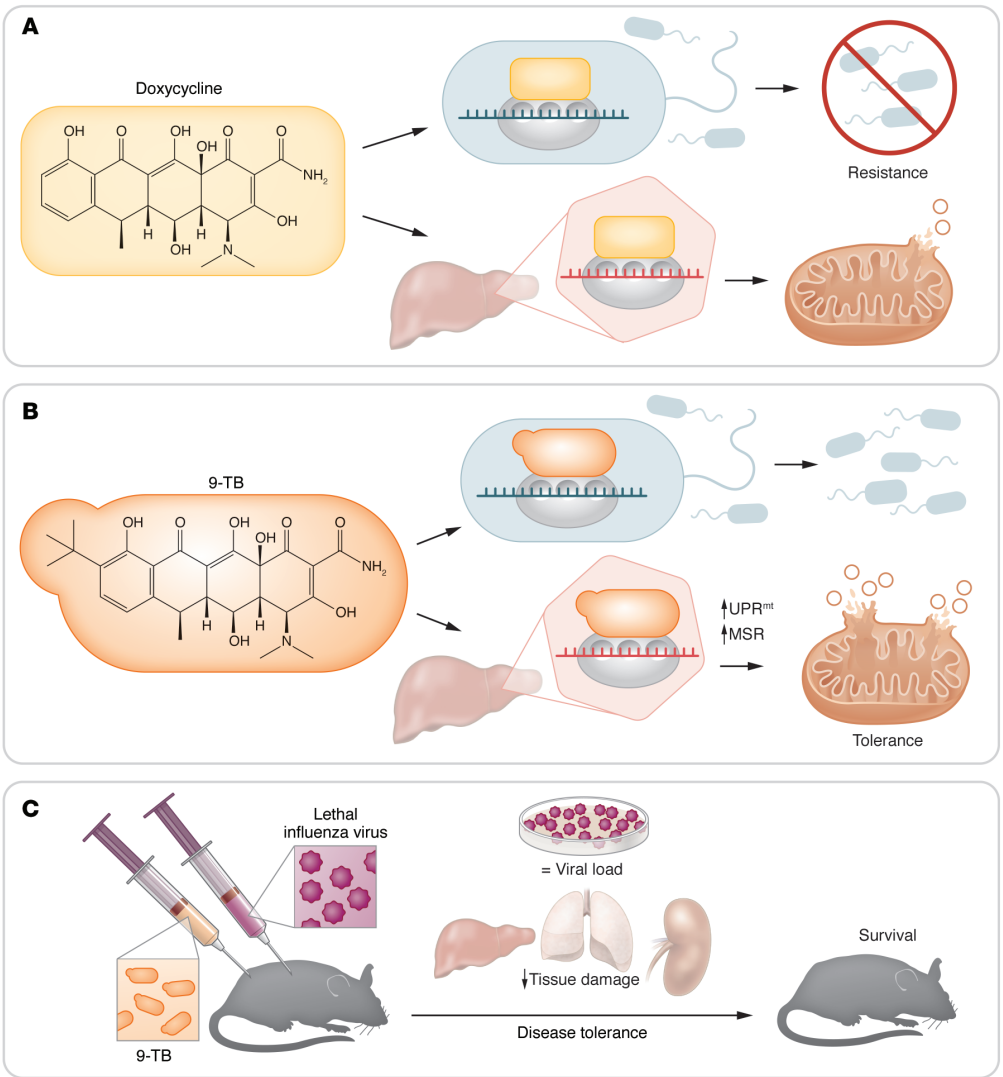

Figure 1. Tetracycline derivatives with minimal antimicrobial activity have increased capacity to induce an adaptive mitochondrial stress response and enhance disease tolerance. (**A**) Doxycycline, a prototypical tetracycline antibiotic, blocks bacterial and mitochondrial translation, inducing mild proteotoxic mitochondrial stress, which initiates mitochondrial stress responses. (**B**) 9-*tert*-Butyl doxycycline (9-TB), a doxycycline derivative with a substitution at the C9 position, has minimal antimicrobial activity but shows substantially greater capacity to induce the UPR^{mt} and mitochondrial stress response (MSR) when compared with parental doxycycline. (**C**) Mottis et al. (17) showed that both parental doxycycline and 9-TB improved survival of mice in a model of lethal influenza virus infection, by reducing tissue damage but without affecting viral titers. This finding demonstrates that in addition to the antimicrobial properties of tetracyclines (known as resistance), the effect of this class of antibiotics on the host mitochondria triggers disease tolerance mechanisms in viral infections through activation of MSRs.

(14). More recently, aminoglycosides were shown to enhance host resistance to viral infections independently of their antibacterial effects, in a microbiota-independent manner (15). These antiviral effects are based on an induced interferon (IFN) response and are, therefore, a surprising example of host resistance against viral infection that is enhanced by antibiotics.

Antibiotics, like most other drugs in clinical use, have varying degrees of off-target effects that may account for undesirable side effects. Unexpectedly, some of these off-target effects induce low-level core cellular function perturbations in the host, which may constitute a critical signal to initiate both resistance and disease tolerance mechanisms. For example, quinolones that target the bacterial enzymes DNA gyrase and DNA topoisomerase IV cause low levels of DNA damage to the host, potentially leading to the induction of IFN-stimulated genes (ISGs), as in the case of aminoglycoside antibiotics (15). Additionally, ribosome-targeting antibiotics (RAbos), which include tetracy-

clines, are bacteriostatic because they block bacterial ribosomes but also mildly inhibit host mitochondrial protein synthesis, given the similarity of the host mitoribosome and bacterial ribosomes. Tetracyclines are a group of broad-spectrum antibiotics that share a common chemical structure based on four (tetra-) linearly fused hydrocarbon rings. This tetracycline nucleus can be modified by the attachment of a diverse set of functional groups that shape their properties. They are active against a wide range of microorganisms that include Gram-positive and Gram-negative bacteria, chlamydiota, mycoplasmatota, rickettsiae, and protozoan parasites. They also have extensive applications outside infection, including in dermatologic conditions like acne. Our laboratory has recently found that host inhibition of mitochondrial protein synthesis by tetracycline antibiotics perturbs the electron transport chain (ETC), leading to improved damage repair in the lung in addition to adrenergic and glucocorticoid sensitivity in the liver. These findings explain the microbiome-independent induction of disease tolerance against sepsis models initiated by tetracycline-resistant bacterial infection (16).

Tetracyclines induce disease tolerance against viral infection

In this issue of the *JCI*, Mottis and colleagues take these observations further to demonstrate that tetracyclines also induce disease tolerance (Figure 1) to influenza virus (IFV) infections (17). Using cellular and germ-free mouse models, where tetracyclines decrease oxidative phosphorylation complex activity and ATP concentrations, the authors demonstrated that the prototypical four-ringed tetracycline doxycycline caused a mild mitochondrial stress response that included both type I IFN signaling and an activating transcription factor 4–mediated (ATF4-mediated) integrated stress response (ISR). Doxycycline caused distinct transcriptional responses in the kidney and liver. While the kidney responded with a transcriptional signature pointing to the activation of the ATF4/ISR pathway, including characteristic translation inhibition, the liver induced a type I IFN response with increased expression of ISGs. Using bone marrow–derived macrophages (BMDMs), the authors identified the release of mtDNA from mitochondria, following its perturbation by doxycycline, as the likely trigger for the initiation of the type I IFN response.

To avoid the antibacterial effects of tetracyclines on the host microbiome, Mottis et al. (17) identified several derivatives with minimal antimicrobial activity. In particular, a derivative with a substitution at the C9 position, 9-*tert*-butyl doxycycline (9-TB), retained, and in fact substantially superseded, the effects of the parental doxycycline on induction of mitochondrial unfolded protein response (UPR^{mt}). 9-TB was also much more potent than doxycycline at inducing a mitochondrial stress response (as measured by the capacity to affect mitonuclear protein imbalance) and inducing ISGs in BMDMs. Doxycycline and 9-TB were effective at increasing survival in a lethal IFV infection model when given preventively. They did not affect the viral titers, a fact that points to their capacity to induce disease tolerance, not resistance mechanisms. Critically, while doxycycline affected the gut microbiome as expected, decreasing its bacterial species diversity, 9-TB did not affect the microbiome composition. Moreover, 9-TB was also capable of decreasing the severity of infection and delaying mortality when administered therapeutically (Figure 1). The authors further showed that disease tolerance to IFV infection correlated with the induction of genes associated with lung epithelia and cilia function. In addition, 9-TB (to a greater extent than doxycycline) downregulated genes with roles in inflammatory and immune responses in the lung, liver, and kidney, possibly limiting tissue damage resulting from an excessive inflammatory response to infection. These findings agree with our demonstration that RAbos impair T cell effector function and ameliorate autoimmunity by blocking mitochondrial protein synthesis (18) because T cells may often cause collateral tissue damage.

Going forward, many exciting questions remain. One is how core cellular function perturbations leading to resistance or disease tolerance are sensed. In the case of tetracyclines it is tempting to speculate that inhibiting mitochondrial protein synthesis perturbs the ETC and decreases ATP concentration, potentially leading to the initiation of UPR^{mt}. This possibility is based on the recent finding that ATP is a strong hydrotrope with the ability to prevent the formation of, and dissolve already formed, protein aggregates (19). Alternatively, tetracycline-induced inhibition of mitochondrial protein synthesis may cause an altered stoichiometry of the ETC complex components that are encoded by nuclei and mitochondria, constituting a signal that is sensed and transduced by unknown factors. A second category of questions will emerge from the systematic investigation of the types of infections that may benefit from the disease tolerance–inducing properties of tetracyclines. Different groups of pathogens impose specific types of tissue damage and are antagonized by appropriate nonoverlapping immune effector responses. Each one of these effector mechanisms comes with its own specific immunopathology and requires unique disease tolerance processes to resolve each pathogen-specific type of tissue damage. Of course, tetracyclines are just the tip of the iceberg. Many other classes of immunomodulatory antibiotics cause their own types of perturbations to cellular processes and organelles. The mechanistic study of their effects is likely to reveal fundamental biological insights into the regulation of organismal homeostasis by stress responses. This knowledge may allow us to harness antibiotic effects for therapeutic strategies against infection and other conditions that progress with inflammation and substantial tissue damage and loss of function, including autoimmune, neurodegenerative, and cardiovascular diseases.

Conclusions and clinical implications

The study by Mottis et al. (17) substantially adds to our understanding of the mechanisms of tetracycline-induced disease tolerance and extends the effects to viral infections, potentially opening the possibility of using tetracycline derivatives as adjuvants for viral infection treatment. Critically, this work demonstrates that the antimicrobial (resistance) activity and the effects on host mitochondria (disease tolerance) of tetracyclines can be dissociated

by the identification of potent tetracycline derivatives like 9-TB, which retain minimal antimicrobial activity but substantially increase effects on mitochondrial function with the potential to induce disease tolerance and minimize tissue damage.

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Trends in
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Spotlight

Ketogenesis favors oxidative phosphorylation to promote disease tolerance

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Luís F. Moita[1,*]

Pseudomonas aeruginosa **is an opportunistic pathogen of great medical relevance, although the mechanisms involved in chronic *P. aeruginosa* infection are unclear. Tomlinson *et al.* have now shown that systemic and local pathogen-induced ketone bodies (KBs) select strains that preserve respiratory integrity by failing to substantially increase glycolysis, which drives immunopathology resulting from resistance mechanisms.**

The resolution of a severe infection requires the synergy of two complementary, evolutionarily conserved, defense strategies to minimize the negative impact on host fitness: resistance and disease tolerance [1]. Resistance targets pathogens directly for elimination and relies on the activation of innate and adaptive immune response mechanisms [2]. Disease tolerance is critical for survival and organ function preservation by limiting and repairing tissue damage caused both by pathogens and the collateral damage caused by resistance mechanisms (immunopathology) [2]. A critical distinction between the two defense mechanisms is that, compared with resistance, disease tolerance does not target the pathogen directly [2]. These two defense strategies rely on mostly distinct transcriptional [3] and metabolic programs [4]; while the programs required for resistance are mostly based on anabolic processes, disease tolerance mechanisms mostly require catabolic pathways, such as oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) [4]. In an acute infection, for evolutionary reasons, lowering the pathogen burden constitutes the initial priority [4]. Therefore, the host favors the elimination of the pathogen (resistance), often at the expense of disease tolerance, risking organ failure resulting from substantial tissue damage in severe infection.

Microorganism-associated molecular patterns (MAMPs) representative of different groups of pathogens are recognized by pattern recognition receptors (PRRs) [1] to initiate innate immune responses. Their engagement leads to the activation of effector mechanisms tailored to each pathogen group and to the generation of long-lasting adaptive immunity [1]. For example, lipopolysaccharide (LPS) is a representative MAMP of Gram-negative bacteria and acts as a ligand for Toll-like receptor 4 (TLR4), the activation of which leads to effector mechanisms that rely on metabolic reprogramming, especially the substantial increase in aerobic glycolysis. Such effector mechanisms are critical for pathogen control and elimination, but often come with substantial tissue damage, organ dysfunction, and even failure. Therefore, disease tolerance is required to limit the consequences of tissue damage and restore organ function.

While the mechanisms of resistance have received considerable attention, and we currently have a detailed cellular and molecular understanding of a wide range of processes, the local and systemic molecular mechanisms that support disease tolerance are only beginning to be explored. Generally, aerobic glycolysis is key to resistance mechanisms mediated by immune effector cells activated by infection, which require anabolic pathways for biosynthesis and proliferation. By contrast, disease tolerance globally requires OXPHOS and FAO [4], except when anabolic programs are necessary for extensive tissue damage repair, which requires cell proliferation to substitute lost cells [4]. KBs are small metabolites endogenously produced in response to several physiological states, including fasting and starvation, and are also known to have direct cytoprotective properties. These can result from the inhibition of several inflammatory pathways as a consequence, for example, of the activation of the β-hydroxybutyrate (BHB) receptor GPR109A [5] and of the inhibition of the NLRP3 inflammasome [6]. Accordingly, BHB concentrations may predict survival in severe infection [7].

In a recent issue of *Cell Metabolism*, Tomlinson *et al.* [8] demonstrate that ketogenesis promotes disease tolerance to *Pseudomonas aeruginosa* pneumonia. *P. aeruginosa* is an opportunistic pathogen that causes acute pulmonary infection, possibly resulting in extensive tissue damage in the lung and consequent loss of function. In patients with cystic fibrosis (CF), it is the leading cause of morbidity and mortality due to its chronic persistence in the lung. The authors demonstrate that ATP synthetase activity in airway cells is inhibited by the surface LPS of *P. aeruginosa*, effectively changing their steady-state metabolic activity from OXPHOS to a predominantly aerobic glycolysis metabolic pattern, supported by the increased expression of glycolytic genes, including that encoding hexokinase 2 (*HK2*). This metabolic switch results in lower levels of energy production and the release of airway-damaging proinflammatory cytokines. In a mouse model of pneumonia initiated by a laboratory strain of *P. aeruginosa* (PAO1), the reduction in energy production resulting from OXPHOS dysfunction activates liver ketogenesis. Liver-produced KBs (BHB) circulate to the lung, where they act on *P. aeruginosa* and select strains that are unable to display surface LPS. This process is accomplished by KBs that

suppress several molecular processes required for the production, assembly, and exposure of LPS by *P. aeruginosa.*

A second component for the development of disease tolerance to *P. aeruginosa* occurs locally, where this pathogen uses the immunometabolite itaconate to drive the expression of ketogenic genes, specifically in lung fibroblasts. The local (lung) increase in KBs further contributes to the selection of less virulent strains, which allow for host–*P. aeruginosa* coexistence, based on the maintenance of alveolar integrity resulting from a decreased inflammatory tissue damage environment and OXPHOS preservation.

This work suggests that KBs are metabolic messengers with a role in the promotion of disease tolerance by selecting pathogen strains that have limited tissue-damaging capabilities (Figure 1). By limiting the tissue damage *P. aeruginosa* imposes on the lung, they also work to the advantage of the pathogen because the host not only is able to survive the acute infection, but also decreases the vigor of the immune response (i.e., resistance), allowing for the establishment of chronic infection.

Notably, while the authors focused their efforts on characterizing the disease tolerance effects of KBs, their results are also clear in the demonstration that KB-adapted strains of *P. aeruginosa*, both *in vivo* and *in vitro*, as well as clinical isolates from patients with CF, proliferate substantially less and impose considerably lower bacterial burdens of infection, at least in the mouse model under study. This is of potential high relevance because it suggests that KBs not only promote disease tolerance, but also dramatically increase resistance. In this case, resistance may be increased not by enhancing immune effector cell efficacy, but by directly acting on the pathogen, as some classes of antibiotic do, to limit its proliferation. Therefore, at least in the case of the

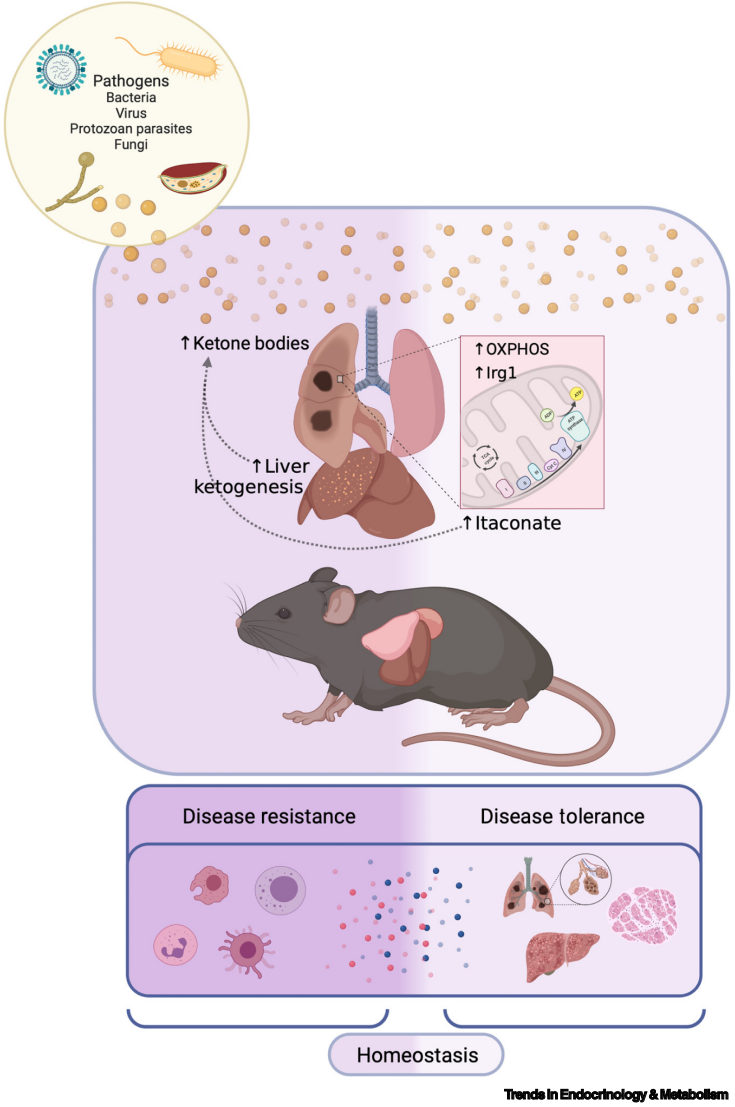

Figure 1. Role of ketone bodies (KBs) in the promotion of disease tolerance to *Pseudomonas aeruginosa.* Decreased energy production in the lung resulting from *P. aeruginosa* infection activates liver ketogenesis. Local lung itaconate drives the expression of ketogenic genes in lung fibroblasts, further increasing the lung concentrations of KBs. Liver and lung-produced KBs promote disease tolerance by selecting *P. aeruginosa* strains that cause less immunopathology and preserve energy production and alveolar barrier integrity. Achieving homeostasis following a severe infection depends on a balance of resistance and disease tolerance mechanisms. Figure created using BioRender (biorender.com).

mouse model of *P. aeruginosa* infection, increased survival may be the result of the dual effect of KBs promoting resistance and disease tolerance.

The authors did not explore the molecular mechanisms by which KBs target several processes necessary for LPS display on the surface of *P. aeruginosa*, certainly an exciting question for the continuation of this line of research. Another issue that deserves attention is the question of how a local lung infection by *P. aeruginosa* drives liver ketogenesis. While the authors attribute this effect to the systemic release of LPS, it appears insufficient to explain liver ketogenesis because there is no difference in sickness behavior components when comparing the infection caused by the wild-type and the *DlptD* mutant PAO1 strains, which lack a transporter critical for LPS exposure on the surface of the outer bacterial membrane. It is possible that disrupted bioenergetics in the lung generates an as-yet-unknown signal to the liver to initiate ketogenesis. If true, this would be an exciting example of inter-organ communication during infection.

Going forward, it will also be interesting and important to substantiate these findings in patients with CF and to determine the extent to which the results reported here may be at play in the case of other chronic infections. In the case of *P. aeruginosa*, KBs block the display of LPS, a characteristic MAMP for Gram-negative bacteria. It will be important to investigate whether this is also the case for representative MAMPs of other pathogen groups, including Gram-positive bacteria and fungi. It is possible that KBs may be exploited by several groups of pathogens to establish stable host–pathogen interactions leading to chronic infection and possibly increased opportunities for transmission. If this is the case, it might be possible to explore these mechanisms to discover novel therapeutic interventions that may be critical for chronic infection management, including in the specific case of patients with CF.

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Declaration of interests

The authors declare no competing interests.

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